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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/16, 15/19, 15/74, 5/10, 1/21, C07K 14/52, 14/575, 16/24, 16/26, A61K 38/19, 38/22, G01N 33/53 // C12R 1:46

A1

(11) International Publication Number: WO 97/15671

(43) International Publication Date: 1 May 1997 (01.05.97)

(21) International Application Number: PCT/US96/17998

(22) International Filing Date: 23 October 1996 (23.10.96)

(30) Priority Data:
60/005,872 26 October 1995 (26.10.95) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: LUMINAL CHOLECYSTOKININ-RELEASING FACTOR

(57) Abstract

Luminal cholecystokinin-releasing factor (LCRF) is a cholecystokinin (CCK) releasing protein isolated from rat intestinal secretion. Purified LCRF was characterized by molecular weight, partial amino acid sequence and CCK releasing activity as shown in *in vivo* studies of anti-LCRF antibodies in blocking the CCK releasing effect of LCRF. Binding studies demonstrated localization in the duodenum, pancreas and in nerve fibers throughout the pancreas, sensory fibers and cell bodies of the nodose ganglia as well as in sympathetic nerve fibers in the adrenal medulla. LCRF appears to be a neuropeptide present in the enteric, parasympathetic and sympathetic nervous systems, but not in the brain. LCRF-IR is also present in enterocytes at the tips of small intestinal villi. Taken together, the studies indicate that LCRF is a neuropeptide that may have several functions in the gastrointestinal systems and other systems. Immunoaffinity studies using antibodies raised to synthetic $LCRF_{1-6}$ and small intestinal lumen infusion studies indicate LCRF may be the CCK-releasing peptide present in intestinal secretion that mediates negative feedback regulation of pancreatic enzyme secretion and CCK release. LCRF and functionally related species have potential for development for treatment of insulin secretion, gastric and gallbladder emptying and regimens requiring appetite control or suppression.

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DESCRIPTION

LUMINAL CHOLECYSTOKININ-RELEASING FACTOR

This is a continuation-in-part of provisional patent application SN 60/005,872 filed October 26, 1995.

The United States government has rights to use of the present invention relative to research support provided by NIH grants R01 DK-37482, R01 DK-38626 and R01 DK 33850.

1.0 BACKGROUND OF THE INVENTION

1.1 Field of the Invention

The invention relates generally to the field of molecular biology and more particularly to novel polypeptides and compositions comprising novel cholecystokinin-releasing peptides (LCRF) and the genes encoding the peptides. In certain embodiments the invention concerns the use of LCRF and nucleic acid sequences encoding the peptides for producing stimulation of an immune response, for appetite suppression, inhibition of gastric emptying, and for stimulation of insulin secretion.

1.2 Description of the Related Art

Cholecystokinin (CCK) is a peptide hormone located in discrete cells of the upper small intestine and secreted into the blood in response to eating. CCK plays a central role in the physiologic regulation of gallbladder contraction and pancreatic secretion and modulates gastric emptying, intestinal motility and appetite (Liddle, 1989). Because of the central role of CCK in digestion, the mechanisms regulating the release of CCK from discrete endocrine cells in the proximal small intestine have been the subject of considerable investigation, reviewed by Liddle (1995).

A large body of evidence indicates that CCK is a natural satiety agent in animals and humans. Part of the "full", pleasant feeling after a meal, termed "satiety", is clearly related to increased CCK release, and has been demonstrated to occur in many human and animal experiments. Unfortunately, CCK acts within internal organs and nerves to cause these effects, and therefore CCK must be administered intravenously or intramuscularly, or possibly by intranasal administration. Moreover, CCK is not effective orally, since it is subject to digestive processes, and secondly, it would still have to be absorbed intact from the intestinal tract, a complicated event, even if it did survive digestive processes

Dietary proteins or protein digests fail to stimulate CCK release from isolated intestinal mucosal cells, and it has been suggested that other factors are necessary for regulation of CCK secretion (Sharara *et al.*, 1993). In conscious rats and man, CCK release and pancreatic exocrine secretion are inhibited by trypsin, chymotrypsin or elastase in the proximal small intestine. This has led to the notion that CCK release may be mediated by a protease-sensitive mechanism (Folsch *et al,* 1987; Slaff *et al,* 1984; Owyang, *et al*, 1986). Based on the potent stimulation of CCK release by diversion of pancreatic juice and bile from the small intestine, Miyasaka and Green (1983) proposed that an intraluminally secreted, trypsin sensitive intestinal factor mediates this response. Such a substance could act as an important feedback regulator of pancreatic enzyme secretion by stimulating CCK release when intestinal free (uncomplexed or uninhibited) protease activity is low, but would be rendered inactive as intestinal free protease activity rises (Green, *et al*, 1972). Subsequently, researchers obtained evidence for an active factor in intestinal washes which stimulated CCK release and pancreatic enzyme secretion in conscious rats (Miyasaka *et al.*, 1989) and in anesthetized rats (Lu *et al.*).

CCK is produced in discrete endocrine cells in the proximal small intestine and is released into the blood stream following a meal. Ingested fats, proteins, and to

a lesser degree, carbohydrates, stimulate CCK release (Marx *et al.*; Fried *et al.*), but the mechanisms underlying the CCK releasing activity of these compounds is unknown.

Studies in rats have demonstrated that diversion of biliary-pancreatic secretions away from the small intestine or infusion of trypsin inhibitors or intact protein into the small intestine strongly stimulates pancreatic enzyme secretion, and this phenomenon is termed "feedback regulation of pancreatic enzyme secretion" (Green *et al.*, 1972; Green *et al.*, 1973). These and later studies show that pancreatic enzyme secretion and CCK release in rats and humans is inhibited by trypsin, chymotrypsin, and elastase in the proximal small intestine (Schneeman *et al.*; Green *et al.*, 1985; Louie *et al.*; Folsch *et al.*; Slaff *et al.*; Owyang *et al.*, 1986).

The hypothesis that protease-dependent feedback regulation of pancreatic enzyme secretion is mediated by an endogenous, intraluminally secreted intestinal peptide was spurred by earlier reports that gastrointestinal peptides appeared in the gut lumen in significant amounts (Uvnas-Wallensten; Lake-Bakaar *et al.*; Chang *et al.*). The origin of luminal peptides was controversial. Some investigators reported that the gut cleared circulating peptides by secreting them into the lumen (Jordan *et al.*; Ayalon *et al.*). On the other hand, Uvnas-Wallensten argued that the immediate source of luminal GI peptides was the corresponding gut endocrine cell (Uvnas-Wallensten), which was described as secreting bi-directionally, *i.e.*, into the lumen and into the circulation *via* diffusion from the interstitial fluid adjacent to basal and lateral parts of the endocrine cell surface.

Feedback regulation of CCK release manifested by dietary protease inhibitors or intact protein (but not by diversion of pancreatic juice) was proposed to be mediated by a cholecystokinin-releasing peptide, monitor peptide (Iwai *et al.*; Fushiki *et al.*), which has been purified from pancreatic juice. Monitor peptide, also known as pancreatic secretory trypsin inhibitor-61 (PSTI-61), is apparently not present in

intestinal secretion (Guan *et al.*). However, two peptides with sequence similarity or identity with monitor peptide have been isolated from pig intestine, although it is not known whether these peptides stimulate CCK release or are secreted intraluminally (Agerbeth *et al.* 1991; Agerbeth *et al.* 1989).

Additionally, Owyang and coworkers (Owyang *et al.* 1990; Herzig *et al.* 1995) have described the purification of a cholecystokinin releasing peptide from porcine intestinal mucosa which stimulates CCK release when infused into the rat intestine. This peptide has been identified as identical to the previously reported peptide diazepam binding inhibitor (DBI).

2. 0 Summary of the Invention

The present invention seeks to address these and other drawbacks inherent in the prior art by providing purified cholecystokinin-releasing polypeptide compositions and methods for treatment of various conditions related to lack of or insufficient regulation of CCK release. The invention relates in particular to a novel polypeptide hormone-like compound, luminal cholecystokinin-releasing factor(LCRF), which was purified from rat intestinal secretions. Immunoaffinity studies using antibodies raised to synthetic LCRF indicate that the polypeptide product isolated and characterized is a CCK-releasing peptide present in intestinal secretion. The properties of the peptide indicate that it mediates "negative feedback regulation" of pancreatic enzyme secretion and CCK release.

LCRF represents one of a new class of regulatory peptides that are secreted intraluminally in the gut and serve an important physiological function in the regulation of metabolic functions that depend on CCK stimulation.

2.1 Novel CCK releasing polypeptides

In an important aspect therefore, the present invention relates to the discovery of a novel CCK-releasing polypeptide isolated from luminal intestinal secretions. The new peptide differs from other known CCK-releasing factors. The partial peptide sequence (SEQ ID NO:1) has little homology with diazepam binding inhibitor (DBI) or other database deposited protein sequences available at the time of the invention.

2.2 LCRF Pharmaceutical Compositions

Another aspect of the present invention includes novel compositions comprising isolated and purified LCRF protein or nucleic acids which encode LCRF protein. It will, of course, be understood that one or more than one CCK-releasing factor gene may be used in the methods and compositions of the invention. The nucleic acid delivery methods may thus entail the administration of one, two, three, or more, homologous genes. The maximum number of genes that may be applied is limited only by practical considerations, such as the effort involved in simultaneously preparing a large number of gene constructs or even the possibility of eliciting an adverse cytotoxic effect.

The compositions will contain a biologically effective amount of the novel peptide or peptides. As used herein a "biologically effective amount" of a peptide or composition refers to an amount effective to stimulate CCK release. As disclosed herein, different peptide amounts are effective, as shown *in vitro* and *in vivo* such as those between about 6 to about 11 mg/kg.

Clinical doses will of course be determined by the nutritional status, age, weight and health of the patient. The quantity and volume of the peptide composition administered will depend on the subject and the route of administration. The precise amounts of active peptide required will depend on the judgment of the practitioner and

may be peculiar to each individual. However, in light of the data presented herein, the determination of a suitable dosage range for use in humans will be straightforward.

The compositions for use in stimulating CCK release in accordance with the present invention will be compositions that contain the full length peptide which has about 70-75 amino acid residues and a molecular weight of about 8136 daltons or functional fragments and variants thereof such as the sequences represent by SEQ ID NO: 1, SEQ ID NO:3 amino acid positions 1-6, 7-23, or 22-37 of SEQ ID NO:1. The term "a peptide" or "a polypeptide" in this sense means at least one peptide or polypeptide which includes a sequence of any of the aforementioned structures or variants thereof. The terms peptide and polypeptide are used interchangeably.

In addition to including an amino acid sequence in accordance with SEQ ID NO:1, the peptides may include various other shorter or longer fragments or other short peptidyl sequences of various amino acids. In certain embodiments, the peptides may include a repeat of shorter sequences, for example, SEQ ID NO:3, or additional sequences such as short targeting sequences, tags, labelled residues, amino acids contemplated to increase the half life or stability of the peptide or any additional residue for a designated purpose, so long as the peptide still functions as a CCK releasing agent. Such functionality may be readily determined by assays such as those described herein.

Any of the commonly occurring amino acids may be incorporated into the peptides, including alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. Likewise, any of the so-called rare or modified amino acids may also be incorporated into a peptide of the invention, including: 2-Aminoadipic acid, 3-Aminoadipic acid, beta-Alanine (beta-Aminopropionic acid), 2-Aminobutyric acid, 4-Aminobutyric acid (piperidinic acid), 6-Aminocaproic acid, 2-Aminoheptanoic acid, 2-Aminoisobutyric acid, 3-Aminoisobutyric acid, 2-Aminopimelic acid, 2,4-Diaminobutyric acid, Desmosine, 2,2'-

Diaminopimelic acid, 2,3-Diaminopropionic acid, N-Ethylglycine, N-Ethylasparagine, Hydroxylysine, allo-Hydroxylysine, 3-Hydroxyproline, 4-Hydroxyproline, Isoeesmosine, allo-Isoleucine, N-Methylglycine sarcosine), N-Methylisoleucine, N-Methylvaline, Norvaline, Norleucine and Ornithine.

The inhibitory compositions of the invention may include a peptide modified to render it biologically protected. Biologically protected peptides have certain advantages over unprotected peptides when administered to human subjects and, as disclosed in U.S. patent 5,028,592, incorporated herein by reference, protected peptides often exhibit increased pharmacological activity.

Compositions for use in the present invention may also comprise peptides which include all L-amino acids, all D-amino acids or a mixture thereof. The use of D-amino acids may confer additional resistance to proteases naturally found within the human body and are less immunogenic and can therefore be expected to have longer biological half lives.

Likewise, compositions that make use of CCK-releasing factor encoding genes are also contemplated. The particular combination of genes may be two or more variants of LCRF genes; or it may be such that a CCK-releasing factor gene is combined with another gene and/or another protein such as a cytoskeletal protein, cofactor or other biomolecule; a hormone or growth factor gene may even be combined with a gene encoding a cell surface receptor capable of interacting with the polypeptide product of the first gene.

In using multiple genes, they may be combined on a single genetic construct under control of one or more promoters, or they may be prepared as separate constructs of the same or different types. Thus, an almost endless combination of different genes and genetic constructs may be employed. Certain gene combinations may be designed to, or their use may otherwise result in, achieving synergistic effects on cell growth

and/or stimulation of an immune response. Any and all such combinations are intended to fall within the scope of the present invention. Indeed, many synergistic effects have been described in the scientific literature, so that one of ordinary skill in the art would readily be able to identify likely synergistic gene combinations, or even gene-protein combinations.

It will also be understood that, if desired, the nucleic acid segment or gene encoding a LCRF polypeptide could be administered in combination with further agents, such as, *e.g.*, proteins or polypeptides or various pharmaceutically active agents. So long as the composition comprises a LCRF gene, there is virtually no limit to other components which may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The nucleic acids may thus be delivered along with various other agents as required in the particular instance.

Pharmaceutical compositions prepared in accordance with the present invention find use in several applications, including appetite suppression, stimulation of insulin release and suppression of gastric or gall bladder emptying. Such methods generally involve administering to a mammal a pharmaceutical composition comprising an immunologically effective amount of a LCRF composition. This composition may include an immunologically-effective amount of either a LCRF peptide or a LCRF-encoding nucleic acid composition. Such compositions may also be used to generate an immune response in a mammal.

Therapeutic kits comprising LCRF peptides or LCRF-encoding nucleic acid segments comprise another aspect of the present invention. Such kits will generally contain, in suitable container means, a pharmaceutically acceptable formulation of LCRF peptide or a LCRF-encoding nucleic acid composition. The kit may have a single container means that contains the LCRF composition or it may have distinct

container means for the LCRF composition and other reagents which may be included within such kits.

The components of the kit may be provided as liquid solution(s), or as dried powder(s). When the components are provided in a liquid solution, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

In related embodiments, the present invention contemplates the preparation of diagnostic kits that may be employed to detect the presence of LCRF proteins or peptides and/or antibodies in a sample. Generally speaking, kits in accordance with the present invention will include a suitable LCRF protein or peptide or antibody directed against such a protein or peptide, together with an immunodetection reagent and a means for containing the antibody or antigen and reagent. The components of the diagnostic kits may be packaged either in aqueous media or in lyophilized form.

The immunodetection reagent will typically comprise a label associated with the antibody or antigen, or associated with a secondary binding ligand. Exemplary ligands might include a secondary antibody directed against the first antibody or antigen or a biotin or avidin (or streptavidin) ligand having an associated label. Of course, as noted above, a number of exemplary labels are known in the art and all such labels may be employed in connection with the present invention. The kits may contain antibody-label conjugates either in fully conjugated form, in the form of intermediates, or as separate moieties to be conjugated by the user of the kit.

The container means will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the antigen or antibody may be placed, and preferably suitably aliquoted. Where a second binding ligand is provided, the kit will also generally contain a second vial or other container into which this ligand

or antibody may be placed. The kits of the present invention will also typically include a means for containing the antibody, antigen, and reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

2.3 LCRF Antibodies

In another aspect, the present invention contemplates an antibody that is immunoreactive with a polypeptide of the invention. An antibody can be a polyclonal or a monoclonal antibody. In a preferred embodiment, an antibody is a monoclonal antibody. Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Howell and Lane, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for LCRF may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of LCRF can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against LCRF. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

To obtain monoclonal antibodies, one would also initially immunize an experimental animal, often preferably a mouse, with a LCRF composition. One would

then, after a period of time sufficient to allow antibody generation, obtain a population of spleen or lymph cells from the animal. The spleen or lymph cells can then be fused with cell lines, such as human or mouse myeloma strains, to produce antibody-secreting hybridomas. These hybridomas may be isolated to obtain individual clones which can then be screened for production of antibody to the desired LCRF peptide.

Following immunization, spleen cells are removed and fused, using a standard fusion protocol with plasmacytoma cells to produce hybridomas secreting monoclonal antibodies against LCRF. Hybridomas which produce monoclonal antibodies to the selected antigens are identified using standard techniques, such as ELISA and Western blot methods. Hybridoma clones can then be cultured in liquid media and the culture supernatants purified to provide the LCRF-specific monoclonal antibodies.

It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and Western blot methods, as well as other procedures which may utilize antibody specific to LCRF epitopes.

Additionally, it is proposed that monoclonal antibodies specific to the particular chemokine may be utilized in other useful applications. For example, their use in immunoabsorbent protocols may be useful in purifying native or recombinant LCRF species or variants thereof.

In general, both poly- and monoclonal antibodies against LCRF may be used in a variety of embodiments. For example, they may be employed in antibody cloning protocols to obtain cDNAs or genes encoding LCRF or related proteins. They may also be used in inhibition studies to analyze the effects of LCRF in cells or animals. Anti-LCRF antibodies will also be useful in immunolocalization studies to analyze the distribution of LCRF during various cellular events, for example, to determine the cellular or tissue-specific distribution of the LCRF peptide under different physiological

conditions. A particularly useful application of such antibodies is in purifying native or recombinant LCRF, for example, using an antibody affinity column. The operation of all such immunological techniques will be known to those of skill in the art in light of the present disclosure.

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2.4 LCRF Compositions and Appetite Suppression

LCRF has distinct advantages as an appetite suppressant and thus as a potential tool in the arsenal of weight management. Unlike CCK, LCRF may be administered orally, thus providing a simple method of treating patients with minimal inconvenience or discomfort.

Effects on gastric emptying may also be an important contributor to satiety and part of the effect of LCRF on satiety may be through its effects to delay gastric emptying.

Once the peptide agent reaches the duodenum, it is subject to digestion by the pancreatic digestive enzymes. LCRF is normally secreted into the lumen of the duodenum and survives intact, if food protein or dietary protease inhibitors are present to protect the peptide from pancreatic digestive enzymes. Orally effective formulations of LCRF could best be taken with meals, and the meal protein would further protect the peptide agent in the intestine. Similarly, a formulation containing a protease inhibitor, such, for example, as potato protease inhibitor II (POT II) or soybean protease inhibitor, along with the peptide agent, may be added to increase the survival of the peptide agent and thus effectiveness in the intestine. For example, oral administration of the peptide hormone, vasopressin, accompanied with a protease inhibitor, Trasylol, resulted in sufficient hormone surviving intestinal digestion to be absorbed in effective amounts (Franco-Saenz *et al.*, 1979).

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Since LCRF is active from the luminal side of the intestine, it is believed necessary only to deliver it safely to the duodenal lumen; it is not necessary to facilitate its absorption. Thus oral preparations will be preferable in most cases.

Orally administered LCRF may be used to stimulate CCK secretion. Should the LCRF be pepsin-sensitive, it may be administered in enterically protected formulations so that it is freed in the small intestine. Alternatively, it may be administered with pepsin inhibitors, inhibitors of stomach acid secretion or antacids of traditional types. LCRF may be made more resistant to digestion by modifying its amino acids, for example, by substituting homoarginine for arginine or replacing one or both lysines. Because LCRF is trypsin-sensitive, fragments of LCRF in the vicinity of one of the lysines or the arginine should retain biological cholecystokinin-releasing or other activities. Amino acid modifications or substitutions with whole or fragmented LCRF are expected to provide more easily prepared and/or digestion-resistant substances.

2.5 LCRF Compositions and Insulin Secretion

LCRF compositions are contemplated to be useful for the stimulation of insulin secretion. CCK has been demonstrated to potentiate amino acid-induced insulin secretion. Therefore, in conditions in which insulin secretion is deficient, such as type I or II diabetes mellitus, CCK may be useful, and therefore a CCK-releasing peptide that is orally active, such as LCRF, will be valuable. In addition, CCK can reduce elevated blood sugar levels after eating a meal by delaying gastric emptying, and can increase small and large intestinal motility. When the above uses for LCRF are described, it is understood that this may involve LCRF fragments, derivatives or analogs that retain the desired biological activities.

LCRF is also useful to regulate stomach emptying, a condition that has been shown to be associated with some types of diabetes. CCK is well-established as a

physiological regulator of stomach emptying; specifically, CCK inhibits stomach emptying. Clinical problems with stomach emptying involve both delayed and accelerated stomach emptying. Early stage diabetes of both type I (insulin-dependent) and type II (non-insulin-dependent, or "adult onset"), involve accelerated stomach emptying, which may later change to delayed stomach emptying when the nervous system is damaged by the disease. Deficient CCK release has been implicated in accelerated stomach emptying in type II diabetes (Rushakoff *et al.*, 1993). LCRF, as an oral agent that releases CCK, will be useful to overcome this defect in early stage diabetes to slow the progression of the disease. There is a significant need for this application because of the large number of people with type II diabetes, especially as the Hispanic and Asiatic populations of the United States increase, as they are particularly susceptible to type II diabetes, particularly when they adopt a more calorie-dense, western-type diet.

2.6 LCRF Compositions and Gallbladder Emptying

LCRF may also be used as part of a treatment for gallbladder disease, particularly gallstones. The need for such a medication is quite large, especially among women, Hispanic-Americans, native Americans, and people undergoing very low calorie weight loss programs. Gallstones occur with varying degrees of frequency in North American populations, depending upon gender, age, diet, socioeconomic status, and ethnicity. The risk is several fold higher in women than men (15-40% after age 50 in Caucasian females), and is increased with obesity. Gallstones occur with dramatic frequency during rapid weight loss, as well as in patients on total parenteral nutrition (TPN). In Hispanic-American females over age 60, the incidence is as high as 44%. The highest reported rate in a defined population is 70% in adult female Pima Indians of the American Southwest.

Although the cause of gallstone formation is complex, a common thread is believed to be reduced motility of the gallbladder, resulting in less frequent and less

complete emptying. Even if small gallstones formed, regular and complete emptying would discharge them harmlessly into the duodenum before they got large enough to be clinically relevant. Since cholecystokinin is the major factor emptying the gallbladder, at least some impaired gallbladder emptying is due to insufficient release of CCK to completely empty the gallbladder. The enhanced release of CCK, as by orally administered LCRF, will improve gallbladder emptying in gallstone-prone people, reduce the incidence of gallbladder disease and thus the need for costly clinical intervention.

2.7 Recombinant LCRF Polypeptides

Recombinant versions of a protein or polypeptide are deemed as part of the present invention. Thus one may, using techniques familiar to those skilled in the art, express a recombinant version of the polypeptide in a recombinant cell to obtain the polypeptide from such cells. The techniques are based on cloning of a DNA molecule encoding the polypeptide from a DNA library, that is, on obtaining a specific DNA molecule distinct from other DNAs. One may, for example, clone a cDNA molecule, or clone genomic DNA. Techniques such as these would also be appropriate for the production of the mutacin polypeptides in accordance with the present invention.

2.8 LCRF Genes

As known to those of skill in the art, the original source of a recombinant gene or DNA segment to be used in a therapeutic regimen need not be of the same species as the animal to be treated. In this regard, it is contemplated that any recombinant LCRF gene may be employed in the methods disclosed herein such as the identification of cells containing DNA encoding LCRF or variants of LCRF.

Particularly preferred genes are those isolated from humans. However, since the sequence homology for genes encoding LCRF polypeptides is expected to be conserved

across species lines, equine, murine, and bovine species may also be contemplated as sources, in that such genes and DNA segments are readily available, with the human or murine forms of the gene being most preferred for use in human treatment regimens. Recombinant proteins and polypeptides encoded by isolated DNA segments and genes are often referred to with the prefix "r" for recombinant and "rh" for recombinant human. As such, DNA segments encoding rLCRFs, or rLCRF-related genes, *etc.* are contemplated to be particularly useful in connection with this invention. Any recombinant LCRF gene would likewise be very useful with the methods of the invention.

Isolation of the DNA encoding LCRF polypeptides allows one to use methods well known to those of skill in the art and as herein described to make changes in the codons for specific amino acids such that the codons are "preferred usage" codons for a given species. Thus for example, preferred codons will vary significantly for bacterial species as compared with mammalian species; however, there are preferences even among related species. Shown below are preferred codon usage tables for rat and human. Isolation of rat DNA encoding LCRF will allow substitutions for preferred human codons, although expressed polypeptide product from human DNA is expected to be highly homologous to mammalian LCRF and so would be expected to be structurally and functionally equivalent to LCRF isolated from rat.

TABLE 1

Rattus rattus

Codon	υ[b]	Total #[a]	Codon	υ[b]	Total #[a]	Codon	υ[a]	Total #[a]	Codon	υ[b]	Total #[a]
UUU	15.7	1972	UCU	14.5	1717	UAU	11.8	1393	UGU	9.9	1167
UUC	24.5	2989	UCC	18.7	2212	UAC	18.6	2198	UGC	13.0	1538
UUA	5.1	605	UCA	9.7	1150	UAA	0.8	92	UGA	1.0	122
UUG	12.1	1425	UCG	3.9	461	UAG	0.4	53	UGG	13.7	1612
CUU	11.5	1361	CCU	16.9	1995	CAU	8.7	1031	CGU	5.1	600
CUC	20.9	2462	CCC	19.0	2238	CAC	15.5	1827	CGC	10.6	1251
CUA	6.8	801	CCA	15.5	1833	CAA	9.9	1174	CGA	6.6	779
CUG	41.4	4890	CCG	6.4	754	CAG	32.2	3802	CGG	10.7	1264
AUU	15.7	1848	ACU	12.8	1515	AAU	14.6	1720	AGU	10.3	1221
AUC	28.2	3327	ACC	22.5	2660	AAC	22.5	2656	AGC	17.7	2094
AUA	5.8	686	ACA	14.3	1688	AAA	20.7	2441	AGA	9.9	1163
AUG	23.9	2822	ACG	6.5	763	AAG	37.0	4370	AGG	10.6	1247
GUU	10.0	1176	GCU	20.0	2363	GAU	20.4	2408	GGU	11.8	1396
GUC	16.9	2000	GCC	28.5	3368	GAC	29.0	3419	GGC	23.5	2779
GUA	6.6	777	GCA	13.8	1630	GAA	25.0	2946	GGA	15.5	1828
GUG	31.3	3692	GCG	6.4	753	GAG	41.1	4853	GGG	14.9	1762

Coding GC 52.97 % 1st letter GC 55.24% 2nd letter GC 41.44 % 3rd letter GC 62.22%

[a] Total 118048 codons [b] υ = Frequency per 1000

TABLE 2

Homo sapiens

Codon	υ[b]	Total #[a]	Codon	υ[b]	Total #[a]	Codon	υ[a]	Total #[a]	Codon	υ[b]	Total #[a]
UUU	16.6	72711	UCU	14.0	62953	UAU	12.3	55039	UGU	9.5	42692
UUC	21.4	95962	UCC	17.7	79482	UAC	17.0	76480	UGC	12.8	57368
UUA	6.3	28202	UCA	10.7	48225	UAA	0.7	2955	UGA	1.2	5481
UUG	11.5	51496	UCG	4.4	19640	UAG	0.5	2181	UGG	13.5	59982
CUU	11.7	52401	CCU	16.7	74975	CAU	9.6	43193	CGU	4.6	20792
CUC	19.5	87696	CCC	20.0	89974	CAC	14.6	65533	CGC	11.0	49507
CUA	6.3	28474	CCA	16.2	72711	CAA	11.4	51146	CGA	5.9	26551
CUG	40.6	182139	CCG	6.9	30863	CAG	33.7	151070	CGG	11.3	50682
AUU	15.7	70652	ACU	12.8	57288	AAU	16.6	74401	AGU	11.1	49894
AUC	23.7	106390	ACC	21.1	94793	AAC	21.1	94725	AGC	19.1	85754
AUA	6.7	30139	ACA	14.7	66136	AAA	23.2	104221	AGA	10.8	48369
AUG	22.6	101326	ACG	6.7	30059	AAG	33.9	152179	AGG	10.9	48882

TABLE 2 (continued)

Codon	υ[b]	Total #[a]	Codon	υ[b]	Total #[a]	Codon	υ[a]	Total #[a]	Codon	υ[b]	Total #[a]
GUU	10.6	47805	GCU	18.7	83800	GAU	22.0	98712	GCU	11.2	50125
GUC	15.6	70189	GCC	29.2	130966	GAC	27.0	121005	GGC	24.0	107571
GUA	6.6	29659	GCA	15.3	68653	GAA	27.8	124852	GGA	16.9	75708
GUG	30.0	134750	GCG	7.5	33759	GAG	40.8	182943	GGG	16.7	74859

Coding GC 52.96% 1st letter GC 55.98% 2nd letter GC 42.29% 3rd letter GC 60.60%

[a] Total 4489120
[b] υ = Frequency per 1000

The definition of a "LCRF gene", as used herein, is a gene that hybridizes, under relatively stringent hybridization conditions (see, *e.g.*, Maniatis *et al.*, 1982), to DNA sequences presently known to include cytokine gene sequences. The definition of a "CCK-releasing factor gene", as used herein, is a gene that hybridizes, under relatively stringent hybridization conditions to DNA sequences presently known to include CCK-releasing factor gene sequences.

To prepare a LCRF gene segment or cDNA one may follow the teachings disclosed herein and also the teachings of any of patents or scientific documents specifically referenced herein. One may obtain a rLCRF- or other CCK-releasing factor-encoding DNA segments using molecular biological techniques, such as polymerase chain reaction (PCR™) or screening of a cDNA or genomic library, using primers or probes with sequences based on the above nucleotide sequence. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Patents 4,683,195 and 4,683,202 (herein incorporated by reference). The practice of these techniques is a routine matter for those of skill in the art, as taught in various scientific texts (see *e.g.*, Sambrook *et al.*, 1989), incorporated herein by reference. Certain documents further particularly describe suitable mammalian expression vectors, *e.g.*, U.S. Patent 5,168,050, incorporated herein by reference. The LCRF genes and DNA segments that are particularly preferred for use in certain aspects of the present methods are those encoding LCRF and LCRF-related polypeptides.

It is also contemplated that one may clone further genes or cDNAs that encode a CCK-releasing factor peptide, protein or polypeptide. The techniques for cloning DNA molecules, *i.e.*, obtaining a specific coding sequence from a DNA library that is distinct from other portions of DNA, are well known in the art. This can be achieved by, for example, screening an appropriate DNA library which relates to the cloning of a chemokine gene such as LCRF. The screening procedure may be based on the

hybridization of oligonucleotide probes, designed from a consideration of portions of the amino acid sequence of known DNA sequences encoding related cytokine proteins. The operation of such screening protocols are well known to those of skill in the art and are described in detail in the scientific literature, for example, see Sambrook *et al.*, 1989.

Techniques for introducing changes in nucleotide sequences that are designed to alter the functional properties of the encoded proteins or polypeptides are well known in the art, *e.g.*, U.S. Patent 4,518,584, incorporated herein by reference, which techniques are also described in further detail herein. Such modifications include the deletion, insertion or substitution of bases, and thus, changes in the amino acid sequence. Changes may be made to increase the cytokine activity of a protein, to increase its biological stability or half-life, to change its glycosylation pattern, and the like. All such modifications to the nucleotide sequences are encompassed by this invention.

2. 8.1 LCRF-Encoding DNA Segments

The present invention, in a general and overall sense, also concerns the isolation and characterization of a novel gene, *lcr* which encodes the novel CCK-releasing polypeptide, LCRF. A preferred embodiment of the present invention is a purified nucleic acid segment that encodes a protein that has at least a partial amino acid sequence in accordance with SEQ ID NO:1. Another embodiment of the present invention is a purified nucleic acid segment, further defined as including a nucleotide sequence in accordance with SEQ ID NO:2.

In a more preferred embodiment the purified nucleic acid segment consists essentially of the nucleotide sequence of SEQ ID NO:2 its complement and the degenerate variants thereof. As used herein, the term "nucleic acid segment" and "DNA segment" are used interchangeably and refer to a DNA molecule which has been isolated free of total genomic DNA of a particular species. Therefore, a "purified" DNA or nucleic acid segment as used herein, refers to a DNA segment which contains a

LCRF coding sequence yet is isolated away from, or purified free from, total genomic DNA, for example, total cDNA or human genomic DNA. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified lcr gene refers to a DNA segment including LCRF coding sequences isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, cDNA sequences or combinations thereof. "Isolated substantially away from other coding sequences" means that the gene of interest, in this case *lcr*, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences which encode a *lcr* gene, that includes within its amino acid sequence an amino acid sequence in accordance with SEQ ID NO:1. Moreover, in other particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences which encode a gene that includes within its amino acid sequence the amino acid sequence of a *lcr* gene corresponding to murine *lcr*.

Another preferred embodiment of the present invention is a purified nucleic acid segment that encodes a protein in accordance with SEQ ID NO:1, further defined as a recombinant vector. As used herein the term, "recombinant vector", refers to a vector

that has been modified to contain a nucleic acid segment that encodes a LCRF protein, or a fragment thereof. The recombinant vector may be further defined as an expression vector comprising a promoter operatively linked to said LCRF-encoding nucleic acid segment.

A further preferred embodiment of the present invention is a host cell, made recombinant with a recombinant vector comprising a *lcr* gene. The recombinant host cell may be a prokaryotic cell. In a more preferred embodiment, the recombinant host cell is a eukaryotic cell. As used herein, the term "engineered" or "recombinant" cell is intended to refer to a cell into which a recombinant gene, such as a gene encoding LCRF, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced gene. Engineered cells are thus cells having a gene or genes introduced through the hand of man. Recombinantly introduced genes will either be in the form of a cDNA gene (*i.e.*, they will not contain introns), a copy of a genomic gene, or will include genes positioned adjacent to a promoter not naturally associated with the particular introduced gene.

Generally speaking, it may be more convenient to employ as the recombinant gene a cDNA version of the gene. It is believed that the use of a cDNA version will provide advantages in that the size of the gene will generally be much smaller and more readily employed to transfect the targeted cell than will a genomic gene, which will typically be up to an order of magnitude larger than the cDNA gene. However, the inventors do not exclude the possibility of employing a genomic version of a particular gene where desired.

In certain embodiments, the invention concerns isolated DNA segments and recombinant vectors which encode a protein or peptide that includes within its amino acid sequence an amino acid sequence essentially as set forth in SEQ ID NO:1. Naturally, where the DNA segment or vector encodes a full length LCRF protein, or is

intended for use in expressing the LCRF protein, the most preferred sequences are those which are essentially as set forth in SEQ ID NO:1. It is recognized that SEQ ID NO:1 represents 41 of the 63-70 or so amino acids of the full length protein encoded by the *lcr* gene and that contemplated embodiments include up to the full length sequence and functional variants as well.

The term "a sequence essentially as set forth in SEQ ID NO:1" means that the sequence substantially corresponds to a portion of SEQ ID NO:1 and has relatively few amino acids which are not identical to, or a biologically functional equivalent of, the amino acids of SEQ ID NO:1. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein, as a gene having a sequence essentially as set forth in SEQ ID NO:1, and that is associated with a constitutively-produced CCK-releasing factor in the LCRF family. Accordingly, sequences which have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids which are identical or functionally equivalent to the amino acids of SEQ ID NO:1 will be sequences which are "essentially as set forth in SEQ ID NO:1"

In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that include within their sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:2. The term "essentially as set forth in SEQ ID NO:2," is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:2, and has relatively few codons which are not identical, or functionally equivalent, to the codons of SEQ ID NO:2. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, as set forth in Table 1, and also refers to codons that encode biologically equivalent amino acids.

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3'

sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences which may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

Excepting intronic or flanking regions, and allowing for the degeneracy of the genetic code, sequences which have between about 70% and about 80%; or more preferably, between about 80% and about 90%; or even more preferably, between about 90% and about 99%; of nucleotides which are identical to the nucleotides of SEQ ID NO:2 will be sequences which are "essentially as set forth in SEQ ID NO:2". Sequences which are essentially the same as those set forth in SEQ ID NO:2 may also be functionally defined as sequences which are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NO:2 under relatively stringent conditions. Suitable relatively stringent hybridization conditions will be well known to those of skill in the art and are clearly set forth herein, for example conditions for use with Southern and Northern blot analysis, and as described in Example herein set forth.

Naturally, the present invention also encompasses DNA segments which are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:2. Nucleic acid sequences which are "complementary" are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences which are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:2 under relatively stringent conditions.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared which include a short stretch complementary to SEQ ID NO:2, such as about 10 to 15 or 20, 30, or 40 or so nucleotides, and which are up to 200 or so base pairs in length. DNA segments with total lengths of about 500, 200, 100 and about 50 base pairs in length are also contemplated to be useful.

A preferred embodiment of the present invention is a nucleic acid segment which comprises at least a 14-nucleotide long stretch which corresponds to, or is complementary to, the nucleic acid sequence of SEQ ID NO:2. In a more preferred embodiment the nucleic acid is further defined as comprising at least a 20 nucleotide long stretch, a 30 nucleotide long stretch, 50 nucleotide long stretch, 100 nucleotide long stretch, or at least an 200 nucleotide long stretch which corresponds to, or is complementary to, the nucleic acid sequence of SEQ ID NO:2. The nucleic acid segment may be further defined as having the nucleic acid sequence of SEQ ID NO:2.

An related embodiment of the present invention is a nucleic acid segment which comprises at least a 14-nucleotide long stretch which corresponds to, or is complementary to, the nucleic acid sequence of SEQ ID NO:2, further defined as comprising a nucleic acid fragment of up to 10,000 basepairs in length. A more preferred embodiment if a nucleic acid fragment comprising from 14 nucleotides of SEQ ID NO:2 up to 5,000 basepairs in length, 3,000 basepairs in length, 1,000 basepairs in length, 500 basepairs in length, or 100 basepairs in length.

Naturally, it will also be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NOS:2 and 1. Recombinant vectors and isolated DNA segments may therefore variously include the LCRF coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides which nevertheless include LCRF-coding regions or may encode biologically functional equivalent proteins or peptides which have variant amino acids sequences.

The DNA segments of the present invention encompass biologically functional equivalent LCRF proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency which are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the LCRF protein or to test LCRF mutants in order to examine activity or determine the presence of LCRF peptide in various cells and tissues at the molecular level.

A preferred embodiment of the present invention is a purified composition comprising a polypeptide having an amino acid sequence in accordance with SEQ ID NO:1. The term "purified" as used herein, is intended to refer to a LCRF protein composition, wherein the LCRF protein is purified to any degree relative to its naturally-obtainable state, *i.e.*, in this case, relative to its purity within a eukaryotic cell extract. A preferred cell for the isolation of LCRF protein is a pancreas or intestinal villi cell, however, LCRF protein may also be isolated from patient specimens, recombinant cells, tissues, isolated subpopulations of tissues, and the like, as will be known to those of skill in the art, in light of the present disclosure. A purified LCRF protein composition

therefore also refers to a polypeptide having the amino acid sequence of SEQ ID NO:1, free from the environment in which it may naturally occur.

If desired, one may also prepare fusion proteins and peptides, *e.g.*, where the LCRF coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (*e.g.*, proteins which may be purified by affinity chromatography and enzyme label coding regions, respectively).

Turning to the expression of the *lcr* gene whether from cDNA based or genomic DNA, one may proceed to prepare an expression system for the recombinant preparation of LCRF protein. The engineering of DNA segment(s) for expression in a prokaryotic or eukaryotic system may be performed by techniques generally known to those of skill in recombinant expression. For example, one may prepare a LCRF-GST (glutathione-S-transferase) fusion protein that is a convenient means of bacterial expression. However, it is believed that virtually any expression system may be employed in the expression of LCRF.

LCRF may be successfully expressed in eukaryotic expression systems, however, the inventors contemplate that bacterial expression systems may be used for the preparation of LCRF for all purposes. The cDNA containing *lcr* gene may be separately expressed in bacterial systems, with the encoded proteins being expressed as fusions with ß-galactosidase, avidin, ubiquitin, *Schistosoma japonicum* glutathione S-transferase, multiple histidines, epitope-tags and the like. It is believed that bacterial expression will ultimately have advantages over eukaryotic expression in terms of ease of use and quantity of materials obtained thereby.

It is proposed that transformation of host cells with DNA segments encoding LCRF will provide a convenient means for obtaining an LCRF protein. It is also proposed that cDNA, genomic sequences, and combinations thereof, are suitable for

eukaryotic expression, as the host cell will, of course, process the genomic transcripts to yield functional mRNA for translation into protein.

Another embodiment is a method of preparing a protein composition comprising growing recombinant host cell comprising a vector that encodes a protein which includes an amino acid sequence in accordance with SEQ ID NO:1, under conditions permitting nucleic acid expression and protein production followed by recovering the protein so produced. The host cell, conditions permitting nucleic acid expression, protein production and recovery, will be known to those of skill in the art, in light of the present disclosure of the *lcr* gene.

2.8.2 Gene Constructs and DNA Segments

As used herein, the terms "gene" and "DNA segment" are both used to refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a gene or DNA segment encoding a LCRF polypeptide refers to a DNA segment that contains sequences encoding a LCRF protein, but is isolated away from, or purified free from, total genomic DNA of the species from which the DNA is obtained. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, retroviruses, adenoviruses, and the like.

The term "gene" is used for simplicity to refer to a functional protein or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences and cDNA sequences. "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a CCK-releasing factor gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude

genes or coding regions, such as sequences encoding leader peptides or targeting sequences, later added to the segment by the hand of man.

2.8.3 Recombinant Vectors Expressing LCRF

A particular aspect of this invention provides novel ways in which to utilize LCRF-encoding DNA segments and recombinant vectors comprising *lcr* DNA segments. As is well known to those of skill in the art, many such vectors are readily available, one particular detailed example of a suitable vector for expression in mammalian cells is that described in U. S. Patent 5,168,050, incorporated herein by reference. However, there is no requirement that a highly purified vector be used, so long as the coding segment employed encodes a LCRF protein and does not include any coding or regulatory sequences that would have an adverse effect on cells. Therefore, it will also be understood that useful nucleic acid sequences may include additional residues, such as additional non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

After identifying an appropriate LCRF-encoding gene or DNA molecule, it may be inserted into any one of the many vectors currently known in the art, so that it will direct the expression and production of the LCRF protein when incorporated into a host cell. In a recombinant expression vector, the coding portion of the DNA segment is positioned under the control of a promoter. The promoter may be in the form of the promoter which is naturally associated with a LCRF-encoding gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

In certain embodiments, it is contemplated that particular advantages will be gained by positioning the LCRF-encoding DNA segment under the control of a

recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a *lcr* gene in its natural environment. Such promoters may include those normally associated with other CCK-releasing polypeptide genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the particular cell containing the vector comprising the LCRF gene.

The use of recombinant promoters to achieve protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.*, (1989). The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level or regulated expression of the introduced DNA segment. The currently preferred promoters are those such as CMV, RSV LTR, the SV40 promoter alone, and the SV40 promoter in combination with the SV40 enhancer.

2.9 Methods of DNA Transfection

Technology for introduction of DNA into cells is well-known to those of skill in the art. Four general methods for delivering a gene into cells have been described: (1) chemical methods (Graham and VanDerEb, 1973); (2) physical methods such as microinjection (Capecchi, 1980), electroporation (Wong and Neumann, 1982; Fromm *et al.*, 1985) and the gene gun (Yang *et al.*, 1990); (3) viral vectors (Clapp, 1993; Danos and Heard, 1992; Eglitis and Anderson, 1988); and (4) receptor-mediated mechanisms (Wu *et al.*, 1991; Curiel *et al.*, 1991; Wagner *et al.*, 1992).

2.9.1 Liposomes and Nanocapsules

The formation and use of liposomes is generally known to those of skill in the art (see for example, Couvreur *et al.*, 1991 which describes the use of liposomes and

nanocapsules in the targeted antibiotic therapy of intracellular bacterial infections and diseases). Recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon and Papahadjopoulos, 1988; Allen and Choun, 1987). The following is a brief description of these DNA delivery modes.

Nanocapsules can generally entrap compounds in a stable and reproducible way (Henry-Michelland *et al.*, 1987). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 mm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be are easily made, as described (Couvreur *et al.*, 1984; 1988).

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 mm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

In addition to the teachings of Couvreur *et al.* (1991), the following information may be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

Liposomes interact with cells via four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or *vice versa*, without any association of the liposome contents. It often is difficult to determine which mechanism is operative and more than one may operate at the same time.

2.10 Expression of LCRF

For the expression of LCRF, once a suitable (full-length if desired) clone or clones have been obtained, whether they be cDNA based or genomic, one may proceed to prepare an expression system for the recombinant preparation of LCRF. The engineering of DNA segment(s) for expression in a prokaryotic or eukaryotic system may be performed by techniques generally known to those of skill in recombinant expression. It is believed that virtually any expression system may be employed in the expression of LCRF.

LCRF may be successfully expressed in eukaryotic expression systems, however, it is also envisioned that bacterial expression systems may be preferred for the preparation of LCRF for all purposes. The cDNA for LCRF may be separately expressed in bacterial systems, with the encoded proteins being expressed as fusions with b-galactosidase, ubiquitin, *Schistosoma japonicum* glutathione S-transferase, green fluorescent protein and the like. It is believed that bacterial expression will ultimately have advantages over eukaryotic expression in terms of ease of use and quantity of materials obtained thereby.

It is proposed that transformation of host cells with DNA segments encoding LCRF will provide a convenient means for obtaining LCRF peptide. Both cDNA and genomic sequences are suitable for eukaryotic expression, as the host cell will, of course, process the genomic transcripts to yield functional mRNA for translation into protein.

It is similarly believed that almost any eukaryotic expression system may be utilized for the expression of LCRF, *e.g.*, baculovirus-based, glutamine synthase-based or dihydrofolate reductase-based systems could be employed. However, in preferred embodiments, it is contemplated that plasmid vectors incorporating an origin of replication and an efficient eukaryotic promoter, as exemplified by the eukaryotic vectors of the pCMV series, such as pCMV5, will be of most use.

For expression in this manner, one would position the coding sequences adjacent to and under the control of the promoter. It is understood in the art that to bring a coding sequence under the control of such a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame of the protein between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter.

Where eukaryotic expression is contemplated, one will also typically desire to incorporate into the transcriptional unit which includes LCRF, an appropriate polyadenylation site (*e.g.*, 5'-AATAAA-3') if one was not contained within the original cloned segment. Typically, the poly A addition site is placed about 30 to 2000 nucleotides "downstream" of the termination site of the protein at a position prior to transcription termination.

Translational enhancers may also be incorporated as part of the vector DNA. Thus the DNA constructs of the present invention should also preferable contain one or more 5' non-translated leader sequences which may serve to enhance expression of the gene products from the resulting mRNA transcripts. Such sequences may be derived

from the promoter selected to express the gene or can be specifically modified to increase translation of the RNA. Such regions may also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence (Griffiths, *et al*, 1993).

Such "enhancer" sequences may be desirable to increase or alter the translational efficiency of the resultant mRNA. The present invention is not limited to constructs where the enhancer is derived from the native 5'-nontranslated promoter sequence, but may also include non-translated leader sequences derived from other non-related promoters such as other enhancer transcriptional activators or genes.

It is contemplated that virtually any of the commonly employed host cells can be used in connection with the expression of LCRFg in accordance herewith. Examples include cell lines typically employed for eukaryotic expression such as 239, AtT-20, HepG2, VERO, HeLa, CHO, WI 38, BHK, COS-7, RIN and MDCK cell lines.

It is contemplated that LCRF may be "overexpressed", *i.e.*, expressed in increased levels relative to its natural expression in human cells, or even relative to the expression of other proteins in a recombinant host cell containing LCRF-encoding DNA segments. Such overexpression may be assessed by a variety of methods, including radio-labeling and/or protein purification. However, simple and direct methods are preferred, for example, those involving SDS/PAGE and protein staining or Western blotting, followed by quantitative analyses, such as densitometric scanning of the resultant gel or blot. A specific increase in the level of the recombinant protein or peptide in comparison to the level in natural LCRF-producing animal cells is indicative of overexpression, as is a relative abundance of the specific protein in relation to the other proteins produced by the host cell and, *e.g.*, visible on a gel.

As used herein, the term "engineered" or "recombinant" cell is intended to refer to a cell into which a recombinant gene, such as a gene encoding a LCRF peptide has

been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced gene. Engineered cells are thus cells having a gene or genes introduced through the hand of man. Recombinantly introduced genes will either be in the form of a cDNA gene (*i.e.*, they will not contain introns), a copy of a genomic gene, or will include genes positioned adjacent to a promoter not naturally associated with the particular introduced gene.

It will be understood that recombinant LCRF may differ from naturally produced LCRF in certain ways. In particular, the degree of post-translational modifications, such as, for example, glycosylation and phosphorylation may be different between the recombinant LCRF and the LCRF polypeptide purified from a natural source, such as intestinal secretions

Generally speaking, it may be more convenient to employ as the recombinant gene a cDNA version of the gene. It is believed that the use of a cDNA version will provide advantages in that the size of the gene will generally be much smaller and more readily employed to transfect the targeted cell than will a genomic gene, which will typically be up to an order of magnitude larger than the cDNA gene. However, the inventors do not exclude the possibility of employing a genomic version of a particular gene where desired.

After identifying an appropriate DNA molecule by any or a combination of means as described above, the DNA may then be inserted into any one of the many vectors currently known in the art and transferred to a prokaryotic or eukaryotic host cell where it will direct the expression and production of the so-called "recombinant" version of the protein. The recombinant host cell may be selected from a group consisting of *S. mutans, E. coli, S. cerevisae. Bacillus sp., Lactococci sp., Enterococci sp., or Salmonella sp.* In certain preferred embodiments, the recombinant host cell will have a *rec*A phenotype.

Where the introduction of a recombinant version of one or more of the foregoing genes is required, it will be important to introduce the gene such that it is under the control of a promoter that effectively directs the expression of the gene in the cell type chosen for engineering. In general, one will desire to employ a promoter that allows constitutive (constant) expression of the gene of interest. Commonly used constitutive promoters are generally viral in origin, and include the cytomegalovirus (CMV) promoter, the Rous sarcoma long-terminal repeat (LTR) sequence, and the SV40 early gene promoter. The use of these constitutive promoters will ensure a high, constant level of expression of the introduced genes. The level of expression from the introduced genes of interest can vary in different clones, probably as a function of the site of insertion of the recombinant gene in the chromosomal DNA. Thus, the level of expression of a particular recombinant gene can be chosen by evaluating different clones derived from each transfection experiment; once that line is chosen, the constitutive promoter ensures that the desired level of expression is permanently maintained. It may also be possible to use promoters that are specific for cell type used for engineering, such as the insulin promoter in insulinoma cell lines, or the prolactin or growth hormone promoters in anterior pituitary cell lines.

2.10.1 Enhanced Production of LCRF

One of the problems with LCRF isolated from natural sources is low yields and extensive purification processes. An aspect of the present invention is the enhanced production of LCRF by recombinant methodologies in a bacterial host, employing DNA constructs to transform Gram-positive or Gram-negative bacterial cells. For example, the use of *Escherichia coli* expression systems are well known to those of skill in the art, as is the use of other bacterial species such as *Bacillus subtilis* or *Streptococcus sanguis*.

Further aspects of the invention include high expression vectors incorporating DNA encoding the novel LCRF and its variants. It is contemplated that vectors providing enhanced expression of LCRF in other systems such as *S. mutans* will also be

obtainable. Where it is desirable, modifications of the physical properties of LCRF may be sought to increase its solubility or expression in liquid culture. The *lcr* locus may be placed under control of a high expression promoter or the components of the expression system altered to enhance expression.

In further embodiments, the DNA encoding the LCRF of the present invention allows for the large scale production and isolation of the LCRF polypeptide. This can be accomplished by directing the expression of the mutacin polhpeptide by cloning the DNA encoding the LCRF polypeptide into a suitable expression vector. Such an expression vector may then be transformed into a host cell that is able to produce the LCRF protein. The LCRF protein may then be purified, e.g., by means provided for in this disclosure and utilized in a biologically active form. Non-biologically active recombinant LCRF may also have utility, *e.g.*, as an immunogen to prepare anti-LCRF antibodies.

2.10.3 Cloning of LCRF Gene

In still another embodiment, the present disclosure provides methods for cloning the DNA encoding the LCRF polypeptide. Using methods well known to those of skill in the art, the DNA that encodes the purified LCRF of the present invention may be isolated and purified. For example, by designing a degenerate oligonucleotide comprising nucleotides complementary to the DNA encoding sequence of SEQ ID NO:1, the LCRF-encoding DNA can be cloned from a pancreas cell library.

The DNA sequences disclosed by the invention allow for the preparation of relatively short DNA (or RNA) sequences which have the ability to specifically hybridize to a gene encoding the LCRF polypeptide. Such a gene, is here termed the *lcr* gene and is understood to mean the gene locus encoding the LCRF structural gene. In these aspects, nucleic acid probes of an appropriate length are prepared. Such

probes are typically prespred based on the consideration of the defined amino acid sequence of purified LCRF. The ability of such nucleic acid probes to specifically hybridize to *lcr* gene sequences lend them particular utility in a variety of embodiments. For example, the probes may be used in a variety of diagnostic assays for detecting the presence of *lcr* genes in intestinal mucosal samples; however, other uses are envisioned, including identification of *lcr* gene sequences encoding similar or mutant polhpeptides related to the mutacin. Other uses include the use of mutant species primers, or primers to prepare other genetic constructs

A first step in such cloning procedures is the screening of an appropriate DNA library, such as, in the present case, genomic or cDNA prepared from an appropriate cell library; for example, pancreas cell. The screening procedure may be an expression screening protocol employing antibodies directed against the protein, or activity assays. Alternatively, screening may be based on the hybridization of oligonucleotide probes, designed from a consideration of portions of the amino acid sequence of the protein, or from the DNA sequences of genes encoding related proteins. Another cloning approach contemplated to be particularly suitable is the use of a probe or primer directed to a gene known to be generally associated with, *e.g.*, within the same operon as, the structural gene that one desires to clone. For example, in the case of LCRF, one may wish to use a primer directed to any conserved regions known to be associated with CCK releasing genes.

Another approach toward identifying the gene(s) responsible for the production of LCRF is tolocate genes known to be adjacent to related CCK releasing factor genes. From sequenced loci in genes that encode other CCK releasing peptides, it will be possible to determine if several processing and export enzymes are highly conserved among the lantibiotic producers and share areas of common sequences. A series of oligonucleotide primers complementary to conserved sequences could be used in PCR™ reactons to amplify the intervening sequence, this amplicon could be used as a probe to identify putative transporter genes. PCR™ technology is described

in U.S. Patent No. 4,603,102, incorporated herein by reference. Where such a transporter gene is found to be part of every known CCK releasing peptide gene, the structural gene for LCRF should be nearby and readily identified by a technique known as "chromosome walking".

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3.0 Brief Description of the Drawings

FIG. 1. Effect of intraduodenal infusion of partially purified intestinal LCRF on pancreatic protein and fluid secretion and on plasma CCK levels (insert). The
10 bioactivity of LCRF is blocked by the CCK receptor antagonist, MK329. *Significantly different from NaCl or MK-329 groups (n = 6, unpaired t-test). **Significantly different from NaCl group (insert, n = 6, unpaired t-test).

FIG. 2. Purification of LCRF by reverse phase high pressure liquid
15 chromatography (HPLC).

FIG. 3. High performance capillary electrophoresis (HPCE) of HPLC-purified LCRF.

20 FIG. 4. Effect of an intraduodenal infusion of pure intestinal LCRF on pancreatic protein and fluid secretion. *Significantly different from NaCl and 1 mg groups. †Significantly different from NaCl group (unpaired t-test)

FIG. 5. Effect of immunoaffinity chromatography using a $LCRF_{1-6}$ antiserum
25 on LCRF bioactivity of partially purified LCRF.

FIG. 6. Changes in pancreatic protein and fluid secretion after an intraduodenal injection of purified LCRF or Monitor Peptide (MP). * denotes significantly different from 9 dose for LCRF. † denotes significantly different from 9
30 dose for MP.

FIG. 7. Dose-response relationship between intraduodenal $LCRF_{1-35}$ and pancreatic secretion. Each point represents 6-8 experiments with the dose indicated, using the bioassay rat model (see text). *denotes significantly different from zero dose for LCRF.

FIG. 8. Comparison between intraduodenal (i.d.) vs. intravenous (i.v.) infusion of $LCRF_{1-35}$. Results for upper panel are from the same experiment illustrated in FIG. 2. * denotes significant difference from zero dose.

FIG. 9. Changes in pancreatic protein and fluid secretion after an intraduodenal injection of various subfragments of $LCRF_{1-35}$. * denotes significantly different from zero dose. The only subfragment with significant with significant biological activity was $LCRF_{11-25}$.

FIG. 10. Changes in pancreatic protein and fluid secretion after an intraduodenal injection of rat Diazepam Binding Inhibitor DBI_{1-86} or ODN peptide DBI_{33-50}. * denotes significantly different from zero dose.

FIG. 11. Effect of CCK-receptor blockade with MK329 on $LCRF_{1-35}$-stimulated pancreatic protein (upper panel) and fluid (lower panel) secretion during return of pancreatic juice to the intestine ("Physiological model"). At the arrow, $LCRF_{1-35}$ was infused intraduodenally at 25 µg/hour for 2 hours during the return of 10% of the secreted pancreatic juice to the duodenum. MK329 was infused at 0.5 mg/hour i.v. starting one hour before first basal collection. * denotes significantly different from basal.

FIG. 12. Incremental protein and fluid output in experiments described in legend of FIG. 6. Results demonstrate the stimulation of pancreatic protein and fluid

secretion by $LCRF_{1-35}$ is abolished by the CCK-receptor antagonist MK325. * denotes significantly different compared to NaCl and $LCRF_{1-35}$ + MK329.

FIG. 13. Plasma CCK concentrations in blood samples taken 60 minutes after start of infusion of test compounds in experiment described in legend of FIG. 9, with the addition of studies with $LCRF_{1-6}$.

FIG. 14. Effect of trypsin digestion of $LCRF_{1-35}$ on its CCK-releasing activity. $LCRF_{1-35}$ was incubated with purified bovine trypsin (1 mg/ml) at 37° C for 24 hours. Control LCRF was incubated under the same conditions but without trypsin. Trypsin control was 1 mg/ml trypsin incubated under the same conditions but without $LCRF_{1-35}$. * denotes significantly different from control.

FIG. 15. $LCRF_{1-35}$ stimulation of CCK release from dispersed rat intestinal cells. * denotes significantly different from zero concentration of $LCRF_{1-35}$.

FIG. 16. Effect of anti-LCRF IgG on pancreatic secretory response to 5% peptone infused intraduodenally in absence of pancreatic juice in the intestine. Peptone was mixed with anti-LCRF IgG and infused together into the duodenum. * denotes significantly different from peptone mixed with normal rabbit IgG. Results show that anti-LCRF IgG abolished the pancreatic secretory response to peptone.

FIG. 17. Effect of LCRF antiserum on the pancreatic secretory response to diversion of bile-pancreatic juice from the duodenum. LCRF antiserum or normal rabbit serum (NRS) were infused intravenously as a bolus (0.1 ml) 1 hour prior to diversion of bile-pancreatic juice. Increment of pancreatic protein and fluid output is shown in insert. * denotes significantly different from NRS-infused group.

FIG. 18. Effect of LCRF antiserum on the plasma CCK response to diversion of bile-pancreatic juice from the duodenum. * denotes significantly different from NRS group and group receiving no serum.

FIG. 19. Lack of effect of $LCRF_{1-35}$ on amylase-release from isolated pancreatic acini. CCK-8 stimulated amylase in a dose-related fashion. At similar concentrations $LCRF_{1-35}$ was without effect. The results indicate that $LCRF_{1-35}$ does not stimulate the pancreas directly, but rather indirectly by stimulating CCK release.

FIG. 20. LCRF immunoreactivity (LCRF-IR) in small intestinal villi. FIG. 15A shows intestinal villi stained using LCRF antiserum 2243232 showing LCRF-IR (dark structures and areas) at the tip and structures in the body of the villi. FIG. 15B: intestinal villi following staining where antiserum was preabsorbed with specific antigen (specific antigen control).

FIG. 21. LCRF-IR in enteric nerves of the small intestine. 21A: LCRF-IR (antiserum 22322) in nerve fibers and nerve cell bodies in the myenteric plexus and submucosal neurons of the duodenum. 16B: Specific antigen control.

FIG. 22. LCRF-IR in the nodose ganglia. 22A: Nerve fibers (dark streaks) and nerve cell bodies (dark patches) in the nodose ganglia stained using antiserum 22322. 17B: Specific antigen control.

FIG. 23 LCRF-IR in the adrenal gland. 23A: Nerve fibers (dark streaks) in the adrenal medulla stained using antiserum 22322. 23B. Specific antigen control.

FIG. 24. Western blot of rabbit antisera reactivity against pancreas, stomach muscle and stomach mucosa tissue. FIG. 24A Is a control with normal rabbit serum. FIG. 24B. Is with rabbit polyclonal serum #QPDG.

FIG. 25. Western blot of rabbit antisera reactivity against pancreas, stromal mucosa, stroma muscle, duodenal muscle, duodenal mucosa, abdominal muscle, ileum mucosa, ileum muscle. FIG. 20A is a control with normal rabbit serum. FIG. 20B is with rabbit polyclonal serum #1728.

4.0 Detailed Description of Preferred Embodiments

A novel CCK releasing factor, luminal cholecystokinin releasing factor (LCRF) has been isolated and purified from intestinal secretions. LCRF is active in stimulating CCK release and is found in enterocytes at the tips of small intestinal villi. It has been identified as a putative neuropeptide found in the enteric, parasympathetic and sympathetic nervous systems, but not in the brain. Immunoaffinity studies using antibodies raised against synthetic $LCRF_{1-6}$ and small intestinal lumen infusion studies suggest that LCRF mediates negative feedback regulation of pancreatic enzyme secretion as well as CCK release.

For practical use, the LCRF peptide and active fragments or analogs thereof may be used to stimulate release of CCK in a manner typical of ingested fats and proteins. Unlike these foods, LCRF effects CCK release at virtually zero caloric input since the peptide is many orders of magnitude more potent in releasing CCK. LCRF acts physiologically from within the lumen of the intestine (*i.e.*, not systemically, or blood-borne); thus it can be delivered to its site of action orally. This contrasts to other bioactive peptides used in medical treatment, *e.g.*, insulin and growth hormone, which must be parenterally administered since they act on cells within internal organs or muscles.

Oral delivery of the LCRF peptide may encounter potential premature destruction by stomach acid and/or pepsin, and\or overly rapid destruction in the intestine by trypsin and other pancreatic proteolytic enzymes. Therefore one will wish to consider embodiments of the agent that include ancillary agents inhibiting

these digestive processes. Such agents are available and well-known to those skilled in the art. Potentially useful agents include medications suppressing stomach acid secretion or action (antacids and acid suppressants such as histamine type II receptor antagonists (Tagamet, Zantac, Pepcid), or H^+, K^+ ATPase inhibitors (*e.g.* Prolesec) as well as agents suppressing trypsin activity (*e.g.*, soybean trypsin inhibitor or potato trypsin/chymotrypsin inhibitor (POT II)). Such compounds have already been used in humans.

Additionally, pepsin-resistant analogs of LCRF or smaller peptide fragments possessing LCRF activity may be employed. The practical result of these embodiments would be to have a formulation mimicking the CCK release that food (particularly fat and protein) causes, but lacking the calories. An exemplary preparation might be synthetic LCRF combined with agents to inhibit its digestive destruction, or chemical analogs (or small fragments) of LCRF that resist digestion.

4.1 ELISAs

ELISAs may be used in conjunction with the invention. In an ELISA assay, proteins or peptides incorporating LCRF antigenic sequences are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, it is desirable to bind or coat the assay plate wells with a nonspecific protein that is known to be antigenically neutral with regard to the test antisera such as bovine serum albumin (BSA), casein or solutions of powdered milk. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

After binding of antigenic material to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the antisera or clinical or biological extract to be

tested in a manner conducive to immune complex (antigen/antibody) formation. Such conditions preferably include diluting the antisera with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween®. These added agents also tend to assist in the reduction of nonspecific background. The layered antisera is then allowed to incubate for from about 2 to about 4 hr, at temperatures preferably on the order of about 25° to about 27°C. Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween®, or borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound antigen, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting same to a second antibody having specificity for the first. To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the antisera-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (*e.g.*, incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween®).

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulfonic acid (ABTS) and H_2O_2, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectrum spectrophotometer.

4.2 Epitopic Core Sequences

The present invention is also directed to protein or peptide compositions, free from total cells and other peptides, which comprise a purified protein or peptide which incorporates an epitope that is immunologically cross-reactive with one or more anti-LCRF antibodies.

As used herein, the term "incorporating an epitope(s) that is immunologically cross-reactive with one or more anti-LCRF antibodies" is intended to refer to a peptide or protein antigen which includes a primary, secondary or tertiary structure similar to an epitope located within a LCRF polypeptide. The level of similarity will generally be to such a degree that monoclonal or polyclonal antibodies directed against the LCRF polypeptide will also bind to, react with, or otherwise recognize, the cross-reactive peptide or protein antigen. Various immunoassay methods may be employed in conjunction with such antibodies, such as, for example, Western blotting, ELISA, RIA, and the like, all of which are known to those of skill in the art.

The identification of LCRF epitopes, and/or their functional equivalents, suitable for use in vaccines is a relatively straightforward matter. For example, one may employ the methods of Hopp, as taught in U.S. Patent 4,554,101, incorporated herein by reference, which teaches the identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity. The methods described in several other papers, and software programs based thereon, can also be used to identify epitopic core sequences (see, for example, Jameson and Wolf, 1988; Wolf *et al.*, 1988; U.S. Patent Number 4,554,101). The amino acid sequence of these "epitopic core sequences" may then be readily incorporated into peptides, either through the application of peptide synthesis or recombinant technology.

Preferred peptides for use in accordance with the present invention will generally be on the order of about 5 to about 25 amino acids in length, and more

preferably about 8 to about 20 amino acids in length. It is proposed that shorter antigenic LCRF-derived peptide sequences will provide advantages in certain circumstances, for example, in the preparation of vaccines or in immunologic detection assays. Exemplary advantages include the ease of preparation and purification, the relatively low cost and improved reproducibility of production, and advantageous biodistribution.

It is proposed that particular advantages of the present invention may be realized through the preparation of synthetic peptides which include modified and/or extended epitopic/immunogenic core sequences which result in a "universal" epitopic peptide directed to LCRF and LCRF-related sequences. It is proposed that these regions represent those which are most likely to promote T-cell or B-cell stimulation in an animal, and, hence, elicit specific antibody production in such an animal.

An epitopic core sequence, as used herein, is a relatively short stretch of amino acids that is "complementary" to, and therefore will bind, antigen binding sites on transferring-binding protein antibodies. Additionally or alternatively, an epitopic core sequence is one that will elicit antibodies that are cross-reactive with antibodies directed against the peptide compositions of the present invention. It will be understood that in the context of the present disclosure, the term "complementary" refers to amino acids or peptides that exhibit an attractive force towards each other. Thus, certain epitope core sequences of the present invention may be operationally defined in terms of their ability to compete with or perhaps displace the binding of the desired protein antigen with the corresponding protein-directed antisera.

In general, the size of the polypeptide antigen is not believed to be particularly crucial, so long as it is at least large enough to carry the identified core sequence or sequences. The smallest useful core sequence anticipated by the present disclosure would generally be on the order of about 5 amino acids in length, with sequences on the order of 8 or 25 being more preferred. Thus, this size will generally correspond to the

smallest peptide antigens prepared in accordance with the invention. However, the size of the antigen may be larger where desired, so long as it contains a basic epitopic core sequence.

The identification of epitopic core sequences is known to those of skill in the art, for example, as described in U.S. Patent 4,554,101, incorporated herein by reference, which teaches the identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity. Moreover, numerous computer programs are available for use in predicting antigenic portions of proteins (see *e.g.*, Jameson and Wolf, 1988; Wolf *et al.*, 1988). Computerized peptide sequence analysis programs (*e.g.*, DNAStar® software, DNAStar, Inc., Madison, Wisc.) may also be useful in designing synthetic LCRF peptides and peptide analogs in accordance with the present disclosure.

Syntheses of epitopic sequences, or peptides which include an antigenic epitope within their sequence, are readily achieved using conventional synthetic techniques such as the solid phase method (*e.g.*, through the use of commercially available peptide synthesizer such as an Applied Biosystems Model 430A Peptide Synthesizer). Peptide antigens synthesized in this manner may then be aliquoted in predetermined amounts and stored in conventional manners, such as in aqueous solutions or, even more preferably, in a powder or lyophilized state pending use.

In general, due to the relative stability of peptides, they may be readily stored in aqueous solutions for fairly long periods of time if desired, *e.g.*, up to six months or more, in virtually any aqueous solution without appreciable degradation or loss of antigenic activity. However, where extended aqueous storage is contemplated it will generally be desirable to include agents including buffers such as Tris or phosphate buffers to maintain a pH of about 7.0 to about 7.5. Moreover, it may be desirable to include agents which will inhibit microbial growth, such as sodium azide or Merthiolate. For extended storage in an aqueous state it will be desirable to store the solutions at 4°C, or more preferably, frozen. Of course, where the peptides are stored in a lyophilized or

powdered state, they may be stored virtually indefinitely, *e.g.*, in metered aliquots that may be rehydrated with a predetermined amount of water (preferably distilled) or buffer prior to use.

4.3 Immunoprecipitation

The antibodies of the present invention are particularly useful for the isolation of antigens by immunoprecipitation. Immunoprecipitation involves the separation of the target antigen component from a complex mixture, and is used to discriminate or isolate minute amounts of protein. For the isolation of membrane proteins cells must be solubilized into detergent micelles. Nonionic salts are preferred, since other agents such as bile salts, precipitate at acid pH or in the presence of bivalent cations.

In an alternative embodiment the antibodies of the present invention are useful for the close juxtaposition of two antigens. This is particularly useful for increasing the localized concentration of antigens, *e.g.*, enzyme-substrate pairs.

4.4 Western Blots

The compositions of the present invention will find great use in immunoblot or western blot analysis. The anti-LCRF antibodies may be used as high-affinity primary reagents for the identification of proteins immobilized onto a solid support matrix, such as nitrocellulose, nylon or combinations thereof. In conjunction with immunoprecipitation, followed by gel electrophoresis, these may be used as a single step reagent for use in detecting antigens against which secondary reagents used in the detection of the antigen cause an adverse background. This is especially useful when the antigens studied are immunoglobulins (precluding the use of immunoglobulins binding bacterial cell wall components), the antigens studied cross-react with the detecting agent, or they migrate at the same relative molecular weight as a cross-reacting signal.

Immunologically-based detection methods for use in conjunction with Western blotting include enzymatically-, radiolabel-, or fluorescently-tagged secondary antibodies against the toxin moiety are considered to be of particular use in this regard.

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4.5 Vaccines

The present invention contemplates vaccines for use in both active and passive immunization embodiments. Immunogenic compositions, proposed to be suitable for 10 use as a vaccine, may be prepared most readily directly from immunogenic LCRF peptides prepared in a manner disclosed herein. Preferably the antigenic material is extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle.

15 The preparation of vaccines which contain LCRF peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables. Either as liquid solutions or suspensions: solid forms suitable for solution in, or suspension in, 20 liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of 25 auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

Vaccines may be conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are 30 suitable for other modes of administration include suppositories and, in some cases, oral

formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides: such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably about 1 to about 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10 to about 95% of active ingredient, preferably about 25 to about 70%.

The LCRF-derived peptides of the present invention may be formulated into the vaccine as neutral or salt forms. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and those which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, *e.g.*, the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

Various methods of achieving adjuvant effect for the vaccine includes use of agents such as aluminum hydroxide or phosphate (alum), commonly used as about 0.05 to about 0.1% solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol®) used as an about 0.25% solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between about 70° to about 101°C for a 30-second to 2-minute period, respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of Gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with a 20% solution of a perfluorocarbon (Fluosol-DA®) used as a block substitute may also be employed.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescents, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

4.6 DNA Segments

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a DNA segment encoding a LCRF peptide in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any viral, prokaryotic (*e.g.*, bacterial), eukaryotic (*e.g.*, fungal, yeast, plant, or animal) cell, and particularly those of mammalian cells. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.*, 1989. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter/expression systems contemplated for use in high-level expression include, but are not limited to, the *Pichia* expression vector system (Pharmacia LKB Biotechnology), a baculovirus system for expression in insect cells, or any suitable yeast or bacterial expression system.

In connection with expression embodiments to prepare recombinant proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire peptide sequence being most preferred. However, it will be appreciated that the use of shorter DNA segments to direct the expression of LCRF peptides or epitopic core regions, such as may be used to generate anti-LCRF antibodies, also falls within the scope of the invention. DNA segments that encode LCRF peptide antigens from about 10 to about 100 amino acids in length, or more

preferably, from about 20 to about 80 amino acids in length, or even more preferably, from about 30 to about 70 amino acids in length are contemplated to be particularly useful.

In addition to their use in directing the expression of LCRF peptides of the present invention, the nucleic acid sequences contemplated herein also have a variety of other uses. For example, they also have utility as probes or primers in nucleic acid hybridization embodiments. As such, it is contemplated that nucleic acid segments that comprise a sequence region that consists of at least an about 14-nucleotide long contiguous sequence that has the same sequence as, or is complementary to, an about 14-nucleotide long contiguous DNA segment of SEQ ID NO:2 will find particular utility. Longer contiguous identical or complementary sequences, *e.g.*, those of about 20, 30, 40, 50, 100, 200, (including all intermediate lengths) and even those up to and including about 220-bp (full-length) sequences will also be of use in certain embodiments.

The ability of such nucleic acid probes to specifically hybridize to LCRF-encoding sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of about 14, 15-20, 30, 40, 50, or even of about 100 to about 200 nucleotides or so, identical or complementary to the DNA sequence of SEQ ID NO:2, are particularly contemplated as hybridization probes for use in, *e.g.*, Southern and Northern blotting. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 10-14 and up to about 100 nucleotides, but larger

contiguous complementarity stretches may be used, according to the length complementary sequences one wishes to detect.

The use of a hybridization probe of about 14 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 14 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of about 15 to about 20 contiguous nucleotides, or even longer where desired.

Of course, fragments may also be obtained by other techniques such as, *e.g.*, by mechanical shearing or by restriction enzyme digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as PCR™, by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNA fragments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating LCRF-encoding DNA segments. Detection

of DNA segments *via* hybridization is well-known to those of skill in the art, and the teachings of U.S. Patents 4,965,188 and 5,176,995 (each incorporated herein by reference) are exemplary of the methods of hybridization analyses. Teachings such as those found in the texts of Maloy *et al.*, 1994; Segal, 1976; Prokop, 1991; and Kuby, 1994, are particularly relevant.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate LCRF -encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a means visible to the human eye

or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantitated, by means of the label.

4.7 Biological Functional Equivalents

Modification and changes may be made in the structure of the peptides of the present invention and DNA segments which encode them and still obtain a functional molecule that encodes a protein or peptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the following codon table:

TABLE 3

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the

inventors that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as

governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

4.8 Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into

the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

4.9 Monoclonal Antibodies

Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Harlow and Lane, 1988; incorporated herein by reference).

The methods for generating monoclonal antibodies (mAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, *m*-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

mAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified LCRF protein, polypeptide or peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B-lymphocytes (B-cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately $5 \cdot 10^7$ to $2 \cdot 10^8$ lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse

myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Milstein, 1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1 x 10^{-6} to 1 x 10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B-cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks.

Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the mAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. mAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

4.10 Pharmaceutical Compositions

The pharmaceutical compositions disclosed herein may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or

they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of

storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus ny additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

The composition can be formulated in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Cholecystokinin secretion in rats and humans is inhibited by pancreatic proteases and bile acids in the intestine. It has been hypothesized that the inhibition caused by pancreatic proteases is due to proteolytic inactivation of a cholecystokinin-releasing peptide present in intestinal secretion. To purify this putative secretory peptide, intestinal secretions were collected by perfusing a modified Thiry-Vella fistula of jejunum in awake rats and these secretions were used as starting material. A peptide was concentrated from intestinal secretions by ultrafiltration and by low pressure reverse phase chromatography, and purified by reverse phase high pressure liquid chromatography. Purity was confirmed by high pressure capillary electrophoresis. Fractions were assayed for CCK-releasing activity by their ability to stimulate pancreatic protein secretion when infused into the proximal small intestine of conscious rats.

Partially-purified fractions strongly stimulated pancreatic secretion and cholecystokinin release, and cholecystokinin receptor blockade abolished the pancreatic response. Amino acid analysis and mass spectral analysis showed that the purified peptide has approximately 70 amino acid residues and a mass of about 8136 daltons. The amino acid composition of LCRF is as follows (amino acid/No. of residues): Ala/4; Arg/1; Asp/9; Cys/N.D.; Glu/11; Gly/6; His/1; Ile/2; Leu/5; Lys/2; Met/0; Phe/2; Pro/7; Ser/7; Thr/7; Trp/N.D.; Tyr/2; Val/3 (N.D.= Not determined in analysis). Microsequence analysis of LCRF yielded an amino acid sequence for 41 amino acids, as follows:

STFWAYQPDGDNDPTDYQKYEHTSSPSQLLAPGDYPCVIEV.

When infused intraduodenally, the purified peptide stimulated pancreatic protein and fluid secretion in a dose-related manner in awake rats and significantly elevated plasma CCK levels. Immunoaffinity chromatography using antisera raised to synthetic $LCRF_{1-6}$ indicated that the CCK releasing activity of intestinal secretion was due to a peptide with the above amino acid sequence. These studies demonstrate the first chemical characterization of a luminally-secreted enteric peptide functioning as an intraluminal regulator of intestinal hormone release.

The intraluminal mediator of protease-sensitive feedback regulation of CCK secretion was purified from intestinal secretions collected by perfusing an isolated loop of jejunum in awake rats. Intestinal secretion appeared to be a better source of this factor than intestinal extracts. This may be because intestinal extracts could contain other releasers of CCK that may not be released into the intestinal lumen.

To purify LCRF, intestinal secretions were collected by perfusing a modified Thiry-Vella fistula of jejunum in awake rats and these secretions were used as starting material. The peptide was concentrated from intestinal secretions by ultrafiltration and by low pressure reverse phase chromatography. It was purified by reverse phase high pressure liquid chromatography. Purity was confirmed by high pressure

capillary electrophoresis. Fractions were assayed for CCK-releasing activity by their ability to stimulate pancreatic protein secretion when infused into the proximal small intestine of conscious rats. Partially-purified fractions strongly stimulated pancreatic secretion and cholecystokinin release and cholecystokinin receptor blockade abolished the pancreatic response.

Amino acid analysis and mass spectral analysis showed that the purified peptide has approximately 70 amino acid residues and a mass of 8136 ± 1% daltons. Microsequence analysis of LCRF yielded an N-terminal amino acid sequence for 41 of the amino acids, as follows:

STFWAYQPDGDNDPTDYQKYEHTSSPSQLLAPGDYPCVIEV.

When infused intraduodenally, the purified peptide stimulated pancreatic protein and fluid secretion in a dose-related manner in awake rats and significantly elevated plasma CCK levels. Immunoaffinity chromatography, using antisera raised to synthetic $LCRF_{1-6}$, confirmed that the amino acid sequence described here was that of a CCK-releasing peptide present in intestinal secretion. The present invention demonstrates the first chemical characterization of a luminally-secreted enteric peptide functioning as an intraluminal regulator of intestinal hormone release.

The dose-response studies with purified intestinal LCRF showed a biphasic curve, with the highest dose producing a submaximal pancreatic protein and fluid response. A similar biphasic dose-response curve for CCK release stimulated by monitor peptide was reported by Cuber *et al.* (1990) in studies using isolated, vascularly-perfused rat intestine. These investigators suggested that the biphasic curve may reflect desensitization of receptors on CCK secreting enteroendocrine cells at higher concentrations of the releasing peptide.

The parallel changes in fluid output and protein output in pancreatic juice suggested that LCRF has secretin-releasing activity as well as CCK-releasing activity.

However, pancreatic fluid secretion in the rat during diversion of bile-pancreatic juice is highly dependent upon CCK, as demonstrated by Taguchi *et al.* (1992) who showed that the greatly elevated fluid output in bile-pancreatic juice-diverted rats was nearly abolished by CCK receptor blockade, in parallel with decreased protein output. Because diversion of pancreatic juice in the rat stimulates secretin release, the stimulation of fluid output by the intestinal LCRF may be interpreted as a reflection of increased levels of CCK augmenting fluid secretion stimulated by a background of elevated secretin secretion (Sun *et al.*,1982). This is also consistent with the virtual elimination of the pancreatic fluid response to partially purified LCRF, by the CCK receptor antagonist, MK-329, in the studies presented here.

LCRF is effective for releasing cholecystokinin in the rat at a dose of 3 micrograms (3 mg) delivered intraduodenally. This translates to approximately 10 mg/kg rat. Conservatively, this suggests that an effective dose for CCK release in a 70 kg man would be approximately 1 mg. For effective treatment, it is believed that this is the amount that would have to be available in the intestine (duodenum or jejunum).

Thus, approximately 1 mg of active LCRF should be present in the duodenum to maximally elicit CCK release in a 70 kg human. Without protective measures other than a meal, it would be expected that only approximately 1-2% would survive digestive processes (DiMagno *et al.*, 1986), meaning that 50-100 mg might be required as an effective oral dose. If accompanied by acid secretory suppressants most (70-80%) of the peptide should survive stomach passage, and be delivered into the duodenum, *i.e.*, a dose of 2-3 mg LCRF with Pepcid or Tagamet should be effective, especially if taken with a meal. If the peptide agent is formulated with a pancreatic protease inhibitor and taken with acid suppressant medication, possibly 100% delivery could be expected, (a dose of 1 mg or less of LCRF then being effective). Likewise, if a chemically-modified form of LCRF, resistant to digestion in stomach and intestine, is made, it would be effective at doses of 1 mg or less.

As discussed, for a peptide given orally in an unprotected form, digestion of the peptide in the stomach and intestine could cause large losses of activity. This is analogous to supplementation with orally administered digestive enzymes in pancreatic disease, in which most of the administered enzymes are destroyed in the stomach by acid/pepsin. Neutralization of gastric contents with gastric acid secretory suppressants (*e.g.*, Tagamet, Zantac or Pepcid) prevents gastric inactivation of oral digestive enzyme supplements (DiMagno *et al.*), and a similar protocol will protect orally-administered LCRF formulations as well. Pepcid and Tagamet are now available without prescription, and Zantac is expected to be so in the near future. Additional protective formulations could include enteric coating of microspheres that encapsulate the agent, such that the microspheres do not release their contents until they reach the duodenum. With these measures, it would be expected that 2-3 mg of LCRF taken orally would result in about 1 mg reaching the duodenum. The oral dosage form of LCRF, its active fragments, derivatives or analogs may be in any convenient administrable form such as a solution, suspension, tablet, capsule or others known to those of skill in the art.

5.0 Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.0.1 Materials

Antisera #94113 and #22322 were raised in rabbits at the antibody core facility of CURE and by Quality Controlled Biochemicals, Inc. (Hopkinton, MA) to $LCRF_{1-6}$ and $LCRF_{7-23}$.

Recombinant diazepam binding inhibitor (DBI_{1-86}) was provided by Jens Knudsen (Odense University, Odense, Denmark). DBI 33-50 (ODN) and Gastrin releasing peptide (GRP) were obtained from Peninsula Laboratories Inc. (Belmont, CA). Recombinant Monitor peptide (MP) was prepared as described in Liddle (Liddle *et al.*, 1984).

5.0.2 Methods

5.0.2.1 Tissue preparation

Wistar male rats weighing between 300 and 350 g were fasted overnight. Rats were anesthetized with pentobarbital (Nembutal, Abbott, Chicago, IL). The brain and brainstem were removed from rats perfused with 4% paraformaldehyde. The nodose ganglia with sections of the vagus nerve, esophagus, stomach, duodenum, pancreas and adrenal glands were removed from non-perfused rats and fixed for 1-2 days in ZamboniÆs solution. All tissue was subsequently cryoprotected in 2 changes of 30% sucrose over 2 days. Some of each tissue sample except brain and brainstem was imbedded in egg yolk gel and sectioned into 30 um slices on a sliding microtome for floating section immunohistochemistry. Brain and brainstem were sectioned into 30 um sections by sliding microtome without imbedding in egg yolk gel. Additional

tissue samples were frozen in Tissue-Tek OCT Compound (Miles Inc, Elkhart, IN), sectioned on a cryostat, and thaw-mounted onto Superfrost slides (Fisher Scientific, Pittsburgh, PA) for antigen blocking studies using adjacent sections.

5.0.2.2 Egg gel embedding

Following fixation and cryoprotection all tissues except brain and brainstem were embedded in "Egg gel" prior to floating section immunohistochemistry. Gelatin was prepared at 6% and 12%, 2 hours prior to embedding and stored at 37° C to allow bubbles to dissipate. A layer of 12% gelatin was poured into the mold which was to be used for the embedding and stored flat until hardened. The tissue was soaked in 6% gelatin at 37° C for 15 min then transferred and soaked in 12% gelatin just prior to embedding. Chicken eggs were brought to room temperature before embedding. An egg was cracked and the white decanted. All the white was removed by rolling the yolk on filter paper and the yolk was mixed with 12% gelatin in a 1:1 ratio. The tissue was removed from the 12% gelatin and put into the mold onto the gelatin base. The yolk gelatin mixture was poured over the tissue being careful not to introduce bubbles and cooled in the refrigerator for 15 min. The molds were immersed in cold 4% paraformaldehyde and refrigerated overnight then incubated at room temperature for 24 hours. The tissue block was removed from the mold and floated in 4% paraformaldehyde for several days then floated in 4% paraformaldehyde with 20% sucrose for 2 days.

5.0.2.3 Immunohistochemistry

Free-floating tissue sections underwent six 10 min washes in 0.05 M PBS, a 20 min incubation in 0.10% (v/v) phenylhydrazine (Fisher, Pittsburgh, PA), followed by four additional 10 min washes in 0.05 M KPBS. Tissue sections were then incubated in primary antibody diluted 1:160,000 in 0.05M KPBS with 0.4% (v/v) Triton-X 100 for sixty min at 22° C then for two days at 4° C. Following incubation the tissues underwent six 10 min washes in 0.05 M KPBS. Tissues were incubated in a solution of biotinylated-Goat anti-Rabbit IgG (Vector #BA1000) diluted to 1:600 in 0.05 M KPBS with 0.4% Triton-X 100 at room temperature for 1 hour then rinsed five times for 10 min with 0.05 M KPBS. Avidin and biotin with horseradish peroxidase (HRP, Vector, ABC Elite) was mixed at a ratio of 45 Ál avidin with 45 Ál biotin in 10 ml 0.05 M KPBS with 0.4% Triton-X 100 and then incubated for 30 min at room temperature. The tissue was incubated with the avidin-biotin complex for 1 hour at room temperature. Following incubation the tissue was rinsed 3 times for 5 min with 0.05 M KPBS then 3 times for 5 min with 0.175 M sodium acetate. The chromogen used was 2 mg diaminobenzadine (Fluka, Switzerland), 250 mg Nickel (II) sulfate, 8.3 Ál of 3% hydrogen peroxide, and 10 ml of 0.175 M sodium acetate. Tissue sections were incubated in chromogen for eight to ten min under direct observation. When optimum staining was obtained the reactions were stopped with three 5 min rinses in 0.175 M sodium acetate followed by three 5 min rinses in 0.05 M KPBS. The floating sections were mounted on Superfrost plus slides, counter-stained with neutral red, and dehydrated through a series of alcohol rinses from 50% to 100%. The tissues were cleared with xylene and cover slips mounted with Histomount (Kimberly research, Atlanta, GA).

5.0.2.4 Antiserum characterization

Optimal antiserum concentration for immunohistochemical studies was determined across a 2-log concentration range. Specificity of staining was determined

by pre-absorbing the santiserum solution with interminal $LCRF_{1-35}$ at 150 ÁM or control solution for 1 hr before antiserum was added to the tissue sections. Optimal antiserum dilution for immunohistochemical studies was determined by titration of the primary antibody through a series of dilutions ranging from 1:1,000 to 1:320,000.

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5.02.4.5 Assays

5.0.2.4.5.1 Protein Assay

Protein output in pancreatic juice was measured by determining optical density at 280 nm of samples diluted in 0.01 M Tris buffer (pH 7.8) and expressed as mg/30 min using bovine trypsinogen as standard. Fluid output was measured by Hamilton syringe and estimated to the nearest 0.001 ml.

5.0.2.4.5.2 CCK bioassay

Plasma CCK was determined by a validated bioassay based on amylase release from isolated pancreatic acini. The same preparation was used to test for direct effects of $LCRF_{1-35}$ on pancreatic acini.

5.1 Example 1

LCRF Isolation and Characterization

5.1.1 Isolation

Wistar male rats weighing between 325-375 grams were fasted overnight. Under methoxyflurane anesthesia (Metofane, Pitman-Moore), rats were prepared with

a modified Thiry-Vella fistula of jejunum. The jejunum was transected at two points, 5 cm and 30 cm from the ligament of Treitz. The proximal end of the jejunal fistula was closed and a Silastic infusion cannula was inserted. The distal cut end was brought to the exterior and secured to the peritoneum and subcutaneous fascia. Gut continuity was reestablished by an end-to-end anastomosis (duodenum to the remaining jejunum). Rats were allowed 3 days recovery from surgery before collection of intestinal secretions began. During recovery and between collections, the Thiry-Vella loop was continually perfused at 2 ml/hr for ~14 hr/day with an elemental-type diet (Vital, 0.5 kcal/ml, Ross Laboratories, Columbus, OH). The purpose of the diet infusion was to prevent mucosal atrophy of the isolated loop. The animals were allowed normal rodent chow and water *ad libitum* after surgery. The surgical procedures are standard techniques and are described in (Guan *et al.*, 1990).

Saline (0.15 M NaCl) was infused at 0.5 ml/min for an hour to wash out any diet remaining in the lumen of the fistula, followed by saline at 1.0 ml/min for 5 hours to flush out the intestinal secretions containing the intestinal CCK releasing peptide (300 ml of diluted intestinal secretion per rat per day). The diluted intestinal secretions (intestinal washout) were collected on ice and at the end of the collection period (5 hr), the washout was boiled for 10 minutes, cooled, then filtered through Whatman number 4 filter paper. The washout was stored at 5° C before protein isolation was undertaken..

5.1.2 Purification

In a cold room (5° C), the intestinal washout was filtered through a YM-30 Amicon disc membrane (MW cutoff of 30,000) using a high-output Amicon stirred cell and then concentrated 100-fold using a YM-1 Amicon disc membrane (MW cutoff of 1000). Concentrates were stored at -70°C. The concentrated washout was further concentrated and purified by using a chain of C_{18} Sep-Paks (Millipore, Milford, MA). Five C_{18} Sep-Paks (classic model) were linked together using Silastic

tubing (elution volume ~5 ml). The Sep-Pak chain was conditioned with 100% ethanol, followed by 0.1% acetic acid. The concentrates (100 ml) were loaded onto the Sep-Pak chain. Subsequently, the chain was washed with 0.1% acetic acid. The intestinal CCK releasing peptide was eluted from the Sep-Pak chain by washing the chain with increasing concentrations of ethanol in 0.1% acetic acid. Ethanol extracts were stored at 5° C prior to further purification by HPLC.

The concentrated samples were diluted five fold with 0.1% trifluoroacetate and loaded by repeated 4 ml injections onto a Vydac C-18 reverse phase HPLC column equilibrated in 0.1 % trifluoroacetate. After loading, the column was rinsed with 0.1 % trifluoroacetate, until the absorbance returned to the value before injection. The sample was then eluted with a gradient to 50% acetonitrile containing 0.1 % trifluoroacetate. The absorbances at 220 and 280 nm were monitored, and peaks were collected.

5.1.3 Analysis

The HPLC protein-containing samples were analyzed by High Performance Capillary Electrophoresis (HPCE) to assess sample purity. A 5 ml sample was diluted three-fold with 0.1 M sodium phosphate, pH 2, and placed onto a Beckman 9600 High Performance Capillary Electrophoresis apparatus. The sample was run according to the manufacturers recommended conditions and data analyzed by System Gold Software.

HPCE revealed elution of a single major component (FIG. 3). A contaminant eluting at 20.7 min was less than 1% the area of the major peak. This contaminant was present in buffer controls and thus did not represent a component isolated from the intestinal washings. The eluted material represented a single pure protein.

An aliquot (50 ml) of the HPCE sample was dried under a vacuum. The sample was hydrolyzed with gaseous HCl for 24 hours then dried by vacuum. The hydrolyzed sample was loaded onto an Applied Biosystems automated amino acid analyzer and analyzed in accordance with the manufacturers recommended procedures.

Analysis showed the amino acid composition of LCRF as shown in Table 4

Table 4

amino acid	No. of residues	amino acid	No. of residues
Ala	4	Lys	2
Arg	1	Met	0
Asp	9	Phe	2
Cys	N.D.	Pro	7
Glu	11	Ser	7
Gly	6	Thr	7
His	1	Trp	N.D.
Ile	2	Tyr	2
Leu	5	Val	3

About 7% of the purified LCRF (100 ml) was loaded onto an Applied Biosystems Peptide Sequencer with automatic PTH analysis. Three analyses were performed on two separately purified samples. One sequence analysis gave conclusive residue assignments up to position 41. The other two sequence analyses gave similar results except residue assignment was not conclusive after position 30. The single letter designation for the amino acid sequence determined is as follows:

STFWAYQPDGDNDPTDYQKYEHTSSPSQLLAPGDYPCVIEV (SEQ ID NO:1)

Several small aliquots of LCRF (5-10 ml) were injected by electrospray onto a Sciex quadrapole mass spectrometer operated in the positive mode. Analysis of LCRF detected one mass ion above background values. The mass of LCRF was measured as 8136.5 daltons, indicating that approximately 2/3 of the sequence of LCRF has been determined. LCRF has a molecular size of 8136 daltons ± 1%, as determined by mass spectral analysis. Assuming an average molecular weight based on the composition analyses, the estimated number of amino acid residues is somewhere around 69-73 amino acid residues.

The amino acid composition of LCRF indicates that it contains three basic residues that can represent potential trypsin cleavage sites. Such sites are consistent with the observation that the releasing factor is inactivated by trypsin (Miyasaka *et al.* 1989).

The determined amino acid sequence for the first 2/3 of the LCRF molecule was compared to sequences in a search program that includes databases SWISS-PROT, PIR, GenPept, and GenPept. Closest homologies for sequences of 30 or so amino acids was no greater than about 35% while closest homology for shorter sequences of 5 amino acids or more was about 60%.

5.2 Example 2

Biological Activity of LCRF

5.2.1 Bioassays

An *in vivo* bioassay for CCK-releasing activity was a modification of the methods described by Miyasaka *et al.* (1992). Male Wistar rats were prepared with pancreatic, biliary, duodenal and jugular vein cannulae. In these animals, the pancreatic juice was diverted from the intestine to prevent proteolytic inactivation of the infused peptides and taurocholate was infused i.d. to suppress the high basal CCK

release caused by diversion of pancreatic juice. Two cannulas were inserted into the duodenum for return of bile-pancreatic juice and for infusion of bioactive peptides. A jugular vein cannula was inserted for blood samples for CCK bioassay. During recovery and between experiments, pancreatic juice and bile were collected and continuously returned to the intestine by a servomechanism consisting of a collecting tube in a liquid level detector coupled to a peristaltic pump. During experiments, pancreatic juice was collected and 10% of the collected secretion was returned to the duodenum. This partial pancreatic juice return model has the advantage of maintaining suppression of basal pancreatic secretion, but reduces the threshold for stimulation by trypsin inhibitors and dietary protein. The rationale for using this in the study of $LCRF_{1-35}$ was to lower the threshold for stimulation of pancreatic secretion by the peptide, analogous to trypsin inhibitor infusion under the same conditions.

At 0800 hr on postoperative days 4 - 7, rats were fasted and their pancreatic juice was diverted from the duodenum. Three hours later bile was also diverted and 40 mM sodium taurocholate containing 100 mM sodium bicarbonate was infused intraduodenally at 1 ml/hr for 3 hours to establish a stable pancreatic secretory rate. Samples were then injected intraduodenally and the pancreatic protein and fluid response was calculated by subtracting the output in the last 15-min basal collection period by the output in the first 15-min collection following the injection of the test solution.

In vitro bioassays based on the ability of LCRF to stimulate CCK secretion from isolated intestinal mucosal cells (Bouras *et al.*, Liddle 1995) or FACS-purified cholecystokinin cells (Liddle *et al.* 1992) were established. The *in vitro* preparations responded to CCK releasing factors such as monitor peptide, KCl and LCRF. One or the other of these *in vitro* assays were used along with the described *in vivo* rat bioassay to follow the purification of LCRF from concentrated intestinal washes. The *in vitro* assays were used to confirm the *in vivo* assays.

To verify that CCK was the hormone stimulating the pancreas in the bioassay, the effect of CCK-receptor blockade (MK-329) on the pancreatic secretory responses to intraduodenal infusion of partially purified LCRF was determined. Partially purified LCRF was infused intraduodenally as described above and pancreatic protein and fluid secretion determined following i.v. injection of MK-329 or vehicle. Plasma CCK levels were also measured during vehicle injection experiments to insure that the bioassay was actually measuring the CCK-releasing activity of the preparations.

5.2.2 Bioactivity of LCRF

Fractions (100-200 ml) collected from the HPLC of intestinal washings as described in Example 5.1, were subjected to Speed-Vac evaporation for ~30 minutes to remove the acetonitrile. 1 ml of 0.1% acetic acid was added and the samples were then loaded onto a single C_{18} Sep-Pak. Sep-Paks were washed with 100% ethanol followed by 0.1% acetic acid. After loading , 1.5 ml of 70% ethanol in 0.1% acetic acid was used for elution. Sample volume was reduced by Speed-Vac to approximately 100 ml; 1 ml of saline was added and pH was adjusted to ~6 to 7 with 0.1 N NaOH.

Bioactivity was found in eluents from the C_{18} Sep-Pak chain in both the 40% and 60% ethanol fractions. Reverse phase HPLC of the 60% ethanol fraction yielded a peak with weak bioactivity, but this peak also contained some impurities. Reverse phase HPLC of the 40% ethanol fraction yielded a single peak with absorbance at 220 and 280 nm that was associated with LCRF bioactivity (FIG. 2). Control tubes before and after this peak had no bioactivity. Once the preparation and chromatography conditions were determined, every preparation of LCRF chromatographed (n=6) had bioactivity in the same position as shown in FIG. 2. Differences in preparations included the amount of LCRF purified and the level of contaminants observed in other regions of the chromatogram.

The purified intestinal LCRF was injected intraduodenally at different doses and the pancreatic protein and fluid secretory response was monitored. 1 mg (n = 5), 2 mg (n = 5), 3 mg (n = 2), and 7 mg (n = 2) of pure LCRF or 0.15 M NaCl (n = 5) was slowly injected into the duodenum of the bioassay rats and the changes in pancreatic protein and fluid secretion were monitored. Responses seen with 3 mg and 7 mg were not evaluated statistically due to the small number of injections. The injection of 2 mg of the polypeptide significantly increased pancreatic protein and fluid secretion by 3.5-fold and 3.1-fold, respectively, compared to saline. The results, illustrated in FIG. 4, show that the pancreatic secretory response to the purified intestinal LCRF is dose-related and biphasic, with the highest dose (7 mg) causing a substantially lower response than the maximally-effective dose (3 mg).

Alternatively, concentrated samples containing partially purified LCRF were subjected to Sephadex gel filtration chromatography. The gel filtration increased the specific bioactivity 100-fold compared to samples obtained after chain Sep-Pak separation. *In vivo* and *in vitro* bioassays of this partially purified preparation were conducted as described above. One ml of blood was withdrawn 15 minutes after the injections of LCRF for plasma CCK determinations. Plasma CCK was measured by bioassay as described by Liddle *et al.* (1984). LCRF injections were repeated in the presence of MK-329 (0.5 mg/kg i.v. bolus), a specific CCK-A receptor antagonist (provided by Dr. Victor J. Lotti, Merck Sharp & Dohme, West Point, PA). MK-329 was dissolved in DMSO:Tween 80:saline (1:1:3) and injected i.v. 1 hr before the injection of the partially purified LCRF.

The effect of an intraduodenal infusion of partially purified LCRF on plasma CCK levels and on pancreatic protein secretion was determined. Two hundred mg of LCRF in 1 ml of 0.15 M NaCl or the NaCl alone was slowly injected (~ one minute) into the duodenum of the bioassay rats. One ml of blood was withdrawn 15 minutes after the injections. LCRF injections were repeated the following day during CCK-A

receptor blockade with MK-329. As shown in FIG. 1, LCRF had an effect that significantly increased plasma CCK levels 4.8-fold compared to saline (0.15 M NaCl). The incremental pancreatic protein and fluid responses to LCRF were 4.2-fold and 2.6-fold higher, respectively, than those seen with the infusion of saline. MK-329 completely abolished the pancreatic secretory response to partially purified LCRF. These results provided strong evidence that the factor being purified is a cholecystokinin-releasing peptide, and that the pancreatic secretory responses observed with the bioassay are due to the release of CCK.

5.3 Example 3

Immunoaffinity Experiments

To confirm that the amino acid sequence reported was in fact that of a CCK-releasing peptide, immunoaffinity chromatography studies were done to selectively remove LCRF bioactivity from intestinal washes. These studies determined that the sequence attributed to LCRF was not that of protein contaminant. Polyclonal antibodies raised against several synthetic LCRF fragments was found to specifically bind to LCRF and to block LCRF activity, thus confirming that the sequence determined was that of a CCK-releasing peptide.

Antisera were raised by standard methods in rabbits to synthetic LCRF (N-terminal hexapeptide at positions 1-6 of SEQ ID NO:1) conjugated to KLH. This antisera (LCRF-Ab) or normal rabbit serum (NRS, control), was coupled to Bio-Rad Affi-Gel 10 gel. A LCRF sample obtained from ultrafiltration of rat intestinal washes was applied to the NRS-coupled gel and to the LCRF-Ab-coupled gel and incubated overnight at 4° C. After 16 hr each gel was transferred to a column support and the unbound material was eluted from the column with 1 M NaCl (Elution Step 1). Subsequently, 20 mM HCl was applied to each column with the objective of eluting the material bound to the antibody by disrupting the antibody-antigen interaction (Elution Step 2). Eluents from Step 1 and Step 2 were concentrated using C-18 Sep-

Paks and speed-vac. Eluents were assayed for CCK-releasing activity by stimulation of pancreatic protein secretion in conscious rats. The antisera was also found to selectively bind to some cells and tissues such as the small intestine, stomach, pancreas, nodose ganglion and brain.

Incubation of partially purified LCRF with the antiserum-coupled gel (Effluent from LCRF-Ab Column) significantly decreased the bioactivity of the material recovered off the gel. LCRF was incubated overnight with an immunoaffinity gel (Bio-Rad Affi-gel 10) to which either($LCRF_{1-6}$ antiserum (LCRFAb) or normal rabbit serum (NRS) was coupled. On the following day, unbound material was eluted from the column supports and assayed for LCRF bioactivity (pancreatic protein secretion). The gel coupled to $LCRF_{1-6}$ antibody apparently bound LCRF as indicated by significantly reduced bioactivity eluting from the column, compared to NRS-coupled gel. The control was an equivalent amount of partially purified LCRF preparation which was not applied to affinity gels. In contrast, incubation with the normal rabbit serum-coupled gel (Effluent from NRS Column) did not significantly affect the bioactivity of the material recovered off that gel. The results are illustrated in FIG. 5. When the antibody-antigen interactions on the gels were disrupted and the gels were eluted, significant amounts of LCRF bioactivity eluted from the antiserum-coupled gel, but no LCRF bioactivity eluted from the NRS-coupled gel (results not shown).

Antisera to two different portions of the LCRF molecule were raised in rabbits. These antibodies were shown to neutralize the CCK-releasing effect of LCRF *in vivo*. Rat Brain, nodose ganglia, stomach, pancreas, duodenum and adrenal were prepared and sliced for immunohistochemistry. Optimal antiserum concentration for immunohistochemical studies was determined across a 2-log concentration range. Specificity of staining was determined by pre-absorbing the antiserum solution with the specific LCRF antigen or nothing for 1 hr before antiserum was added to the tissue sections. Binding was localized using an avidin-biotin complex-horse radish

peroxidase secondary antibody system with nickel-diaminobenzadine chromogen. Sections were counter-stained and analyzed by light microscopy.

Concentration-dependent and antigen-specific staining was identified in both the duodenum and pancreas. Staining was observed in the myenteric and submucosal plexus of the duodenum and stomach. Staining was also identified in nerve fibers throughout the pancreas, sensory fibers and cell bodies of the nodose ganglia, and sympathetic nerve fibers in the adrenal medulla. The: immuno-histochemical evidence suggested that LCRF is a neuropeptide that may have several functions in the gastrointestinal system and other systems.

The specificity of the binding was demonstrated by progressive loss of binding with serial dilution, by the absence of staining with nonspecific rabbit primary antibody, and by blocking of the binding with the specific antigen used to immunize the rabbits (FIGS. 20B, 21B, 22B and 23B)). Immunohistochemical staining of adjacent section with antiserum to LCRF-$_{1-6}$ and LCRF$_{7-23}$ in each of the tissue types demonstrated identical staining patterns, although the antiserum to LCRF$_{7-23}$ was superior to the aminoterminal antiserum for immunohistochemistry. These data suggested that the immunohistochemical staining used for localization accurately reflects LCRF distribution *in vivo*.

5.3.1 LCRF localization in the upper intestine and pancreas

LCRF immunoreactivity was identified in nerve fibers within the proximal two-thirds of the small intestinal villi and in enterocytes at the tips of the villi (FIG. 20A and FIG. 20B). Longitudinal and cross-sectional views of the enterocytes demonstrate LCRF immunoreativity (LCRF-IR) within discrete circular structures in the cytoplasm and fibers. Luminal mucus strands contain LCRF-IR but were incompletely blocked with preabsorbed antiserum. Although LCRF-IR mucus strands

appeared to extend from the distal villi, goblet cells were LCRF-IR negative. Enteroendocrine cells were also LCRF-IR negative.

Nerve fibers and nerve cell bodies in the myenteric plexus and submucosal neurons of the duodenum contain LCRF-IR (FIG. 21A and FIG. 21B). Nerve fibers extending into the villi were traced to the submucosal neurons in some instances although the origin of most fibers could not be determined.

LCRF-IR in the stomach was identified in nerve fibers and nerve cell bodies in the myenteric and submucosal plexus. Enterocytes within the gastroesophageal junction also displayed LCRF-IR. In addition, a number of large LCRF-IR nerves coursed along the serosal surface of the stomach antrum. Large LCRF-IR nerve fibers appear to run through the pancreas, and are especially prominent in the interlobular connective tissue. Small immunoreactive nerves were occasionally seen around the periphery of the islets of Langerhans but these were not always observed.

5.3.2 LCRF immunoreactivity in the autonomic nervous system and brain.

The parasympathentic nervous system was investigated through evaluation of the nodose ganglia with the adjacent vagus, and brainstem sections containing the dorsal motor nucleus of the vagus and the nucleus ambiguous. Nerve cells bodies in the nodose ganglia and vagal fibers are LCRF-IR positive (FIG. 22A and FIG. 22B), whereas the motor neurons in the brain stem are LCRF-IR negative. Thus, only the sensory arm of the vagus contains LCRF-IR.

The adrenal gland was used to screen nerves of the sympathetic nervous system. Cells of the adrenal medulla showed weak LCRF-IR staining as well as distinct staining of sympathetic nerve fibers (FIG. 23A and FIG. 23B). However, no LCRF-IR perivascular sympathetic fibers were observed in the adrenal gland, intestine or other tissues.

The central nervous system was evaluated using regularly spaced sagittal sections covering the entire brain. No LCRF-IR was identified in the central nervous system. Thus, LCRF-IR localizes to nerves of the enteric nervous system, the sensory arm of the vagus, and sympathetic fibers of the adrenal gland.

5.4 Example 4

Molecular Cloning of LCRF

The determination of the major portion of the LCRF amino acid sequence allows the relatively straightforward cloning of the encoding DNA, using degenerate primers to probe an appropriate DNA library. The length of the primer is generally a matter of choice but will conveniently be on the order of 15-25 base pairs and could be up to the full length of the determined 41 amino acid sequence. Degenerate primers synthesized from the sequenced N-terminal amino acids of the peptide will be used to produce, by RT-PCR™, a cDNA encoding that segment of LCRF. Once the cDNA is sequenced, primers generated from 3'-end of the cDNA sequence will be used as 5'-primer, along with oligo$(dT)_{16}$ as 3'-primer, to RACE both ends of the transcript in order to produce an intact full-length cDNA of LCRF.

Rapid amplification of cDNA end (RACE)

The 3'-end of LCRF cDNA will be amplified in a 100 ml reaction mixture containing 10 mM Tris-HCl (pH 8.4; at 23°C), 1.5 mM $MgCl_2$, 40 mM KCl, 200 mM of each dNTP, 1 mM each of a primer from the middle of the peptide already sequenced, 2 ml oligo$(dT)_{16}$, and 2 U Taq DNA polymerase. Thirty cycles of amplification will be carried out with denaturation at 94°C for 1 min, annealing at 40°C for 1 min., and extension at 72°C for 1 min, followed by an additional extension at 72°C for 20 min.

To ensure that the 5'-end of the LCRF transcript is fully sequenced, the latter will be reverse transcribed using the P3-primer. The extended primer will be tailed with poly A in a 20 ml reaction mixture containing 50 mM potassium cacodylate, 2 mM $CoCl_2$, 200 mM DTT, 200 mM dATP, and 10 U terminal deoxynucleotidetidyl transferase. The extended primer will be used as template and amplified as for the 3'-end described above, except that primers and first cDNA will be substituted by 0.2 mM oligo$(dT)_{16}$ primer, 0.5 mM of a specific primer obtained from the sequenced 123-bp cDNA, and 2ml of the tailed first strand cDNA. Finally, the overlapping 3'- and 4'-end RACE products will be combined to produce an intact full-length cDNA of LCRF.

Cloning and Sequencing

PCR™ product will be purified and cloned into pVZI plasmid vector via the TA cloning method from Invitrogen. The nucleotide sequences will be determined by the dideoxynucleotide chain termination method, using [a-^{35}S]dATP and the sequenase kit. An alternative to PCR™ cloning would be a traditional plaque hybridization using a probe based on the known amino acid sequence of LCRF and a cDNA library such as obtained from pancreas or brain cells. Once having the full-length cDNA encoding LCRF, the LCRF cDNA will be used to obtain the human version of this peptide. A human version of LCRF expected to be homologous to the rat LCRF would also be obtainable by analogous procedures.

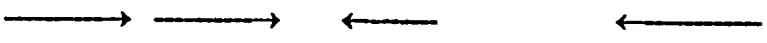
The DNA sequences disclosed in the invention allow for the preparation of relatively short DNA (or RNA) sequences which have the ability to specifically hybridize to *lcr* gene sequences by preparing nucleic acid probes of an appropriate length. Such probes are typically prepared based on the consideration of the defined gene sequence of the LCRF gene or derived from flanking regions of this gene.

In order to clone the gene that encodes LCRF, two complementary strategies are contemplated. One approach has been to use the peptide sequence of SEQ ID NO:1 to design oligonucleotide primers for use in direct cloning by PCR™ (polymerase chain reaction). In a second approach, serological reagents will be used to screen a cDNA library to identify the sequence with immunoreactivity. These two approaches are complementary, but are expected to identify the same DNA or RNA sequence.

Oligonucleotide Approach

From the 41 amino acid sequence determined for the amino terminus of LCRF, the mRNA sequence was predicted and the least degenerate regions were chosen. Six different oligonucleotide primers (from 4 regions) were generated; their sequences and positions as shown.

STFWAYQPDGDNDPTDYQKYEHTSSPSQLLAPGDYPCVIEV (SEQ ID NO:1)



lcrf-5 **lcrf-p** **lcrf-p2** **lcrf-3'**

The sequences of the lcrf oligonucleotides are:

lcrf-5 (inosine) 5'-TT(T/C) TGG GCI TA(T/C) CA(A/G) CCI GA(T/C) GG (SEQ ID NO: 4)

lcrf-5 (degenerate) 5'-TT(T/C) TGG GC(A/C/T) CA(A/G) CC(A/C/T) GA(T/C) GG (SEQ ID NO: 5)

lcrf-p 5'-GA(T/C) AA(C/T) GA(T/C) CCI ACI GA(C/T) TA(T/C) CA (SEQ ID NO: 6)

lcrf-p2 5'-GT(A/G) TG(T/C) TC(A/G) TA(C/T) TT(T/C) TG

SEQ ID NO: 7

lcrf-3' (inosine) 5'-TCI ATI AC(A/G) CAI GG(A/G) TA(A/G) TCI CC

SEQ ID NO: 8

lcrf-3' (degenerate) 5'-TC(T/G/A) AT(C/G) AC(A/G) CA(T/A/G) GG(A/G) GG(A/G) TA(A/G) TCN CC

SEQ ID NO: 9

For each of the outermost oligonucleotides, two different versions were generated, one in which the degenerate positions were filled with inosine and the other in which they contained the appropriate mixture of nucleotides. In general, the LCRF-5' and LCRF-3' oligonucleotides were designed to serve as primers in PCR™, while the internal oligonucleotides were to be used primarily as probes or if necessary, nested primers.

In order to clone the LCRF coding sequence, RNA was prepared from several rat tissues, including intestine, brain, pancreas, stomach, and nodose ganglia. These RNAs were converted to cDNA for use in reverse transcriptase-coupled polymerase chain reaction (RT-PCR™); all were shown to be intact using an HPRT (hypoxanthine phosphoribosyl transferase) control PCR™. Standard PCR™ is employed. In addition, since the primers are highly degenerate, step-down PCR™ is also utilized.

In addition, high molecular weight genomic DNA was isolated from rat liver for use in standard PCR™ amplifications. Several PCR™ products have been obtained and cloned into a pUC for analysis. Next, step-down PCR™ will be used to increase specificity with the DNA PCR™ reactions.

Serological Approach

Prior to generating an expression library, it was necessary to identify a good source of RNA which is likely to contain the LCRF mRNA sequence. In addition, one of more anti-LCRF antibodies that could recognize denatured peptide were required. Thus, to address both issues, Western blots were prepared using protein extracts from several different sources. The protein blots were then incubated individually with 4 different antisera. In the pancreas extract; all 4 antisera detected a band of the same size ~20 kD. Thus, a cDNA expression library will be constructed from pancreas mRNA and screened directly with the polyclonal anti-LCRF reagents. The cDNAs detected will be sequenced to ensure that they contain the appropriate coding information.

The identified LCRF cDNA will be used to clone the full-length cDNA from both rat and human cDNA libraries. The cDNAs will be cloned into expression vectors in order to produce large amounts of LCRF for physiological analysis. In addition, the LCRF gene will be cloned from human and mouse genomic libraries to further define its regulatory actions. The inventors further contemplate using the murine gene to generate a knock-out mouse deficient for LCRF for use in assessing the biological role of this peptide.

5.5 Example 5

Methods of utilizing the effect of LCRF on CCK Release

LCRF administration is superior to CCK or CCK agonists. This is because LCRF releases endogenous cholecystokinin, which is predominately CCK-58 in blood of humans and dogs. CCK-58 is too large a molecule to synthesize economically for pharmaceutical purposes. However, CCK-58 released by LCRF would be preferable to the form of CCK approved for medical use, *i.e.*, injected CCK-8, because the former has a longer half-life and preferable receptor binding characteristics compared to CCK-8. Likewise, potential CCK agonists, peptide as well as non-peptide, would be less physiological than endogenous CCK.

The activity of LCRF indicates its utility in controlling CCK release and thus providing treatment methods for several conditions in which CCK is involved in a regulatory capacity. LCRF and truncated forms and active variants may be synthesized by standard techniques and their ability to release CCK determined *in vitro* and *in vivo*. *In vitro* methods are based on the ability of LCRF active peptides to release CCK from dispersed intestinal mucosal cells or from STC-1 cells, a tumor cell line that secretes CCK in response to CCK-releasing peptides such as monitor peptide, bombesin, as well as LCRF. *In vivo* methods include intraduodenal or intragastric or intravenous infusion of LCRFs.

5.5.1. Oral Pharmaceutical Compositions

Forms in which LCRF may be administered orally

LCRF is a polypeptide, like insulin, so it is subject to digestion in the stomach, by acid/pepsin, and in the small intestine by pancreatic proteases. But, unlike insulin (and CCK itself), LCRF presumably acts on receptors on the luminal side of mucosal cells (CCK-releasing cells) so doesn't have to be absorbed. Insulin would have to be absorbed intact to reach cellular receptors, and this is improbable. This makes LCRF

unique as a regulatory peptide, and makes oral delivery practical whereas for other regulatory peptides (growth hormone, insulin, etc. oral administration is impractical.

Administration of LCRF orally would be practical in a multitude of forms. The compound is heat stable (survives boiling for 10 min, and survives incubation at 37° for 24 hours, with loss of about 20% activity). It is water soluble, and effective at very low concentrations, such as 0.08 mg/kg body weight in the adult rat, given intraduodenally to stimulate CCK release, or 0.15 mg/kg to suppress food intake in neonatal rats, administered intragastrically. Thus as little as 10 mg may effective be orally in a 70 kg human.

Forms in which LCRF can be administered orally:

Powder: As the pure peptide, mixed in a powder vehicle such as dry milk, dry cocoa, sugar, which mixture could then be dissolved in water or other suitable liquid vehicle. In this form, the peptide would be unprotected from gastric or intestinal digestion, as in neonatal rats, and therefore the dose would be expected to be in the range of 10 mg/kg. Although administration of LCRF orally without additional efforts to prevent losses due to inactivation in stomach and intestine may seem inefficient, it is not an important barrier to successful treatment since it can be overcome by simply increasing the dose. This is not dangerous because the excess (wasted) peptide is simply digested like any other protein in the diet.

Such powdered forms would be taken in advance of a meal, to take advantage of the "pre-load" phenomenon, in which giving a small meal 10 or 20 min before a regular meal can markedly reduce the amount of the meal consumed.

Capsule: LCRF can be administered in a capsule such that it can be taken with a meal or before a meal. This would be convenient, whether or not the capsule is coated to resist digestion in the stomach and intestine.

Enteric coated preparations: To reduce the dose of LCRF needed, preparations of LCRF can be in enteric coated capsules, or enteric coated. This technology has been in widespread use in the oral administration of pancreatic enzyme supplements. The preparations permit the encapsulated preparation to survive gastric digestive processes, releasing their contents in the non-acid pH environment of the intestine.

Protease inhibitor preparations: Oral protease inhibitors stimulate CCK release by protecting endogenous LCRF or other endogenous luminal CCK-releasing peptides, according to the hypothesis of Miyasaka et al (1992). Thus, it is logical to consider mixing protease inhibitors, such as POT II, *i.e.*, potato protease inhibitor II , with LCRF to make a preparation that enhances the efficacy of LCRF by protecting it from digestion in the small intestine. POT II (U.S. Pat. No. 5,468,727, the entire disclosure of which is incorporated by reference), stimulates CCK release and inhibits gastric emptying in humans.

In humans these effects presumably occur by protecting an endogenous human versions of LCRF. Thus, POT II could be made into a formulation which included synthetic LCRF and incorporated into a capsule of microencapsulated for protection from gastric acid/pepsin, and this formulation would be expected to survive both gastric and intestinal protease digestive barriers and deliver nearly 100% of the ingested dose of LCRF to the appropriate receptors on the intestinal mucosa. With such a preparation, we predict that as little as 1 mg/70 kg of LCRF would be highly effective in stimulating CCK release in humans, to effect increasing satiety values for foods taken prior to or with the LCRF preparation, to slow gastric emptying and thereby slow glucose absorption and uptake, ameliorating postprandial hyper- and hypo-glycemia and hyperinsulinemia, more complete emptying of the gallbladder to reduce likelihood of stone formation, improved functioning of the gastro-colic reflex which promotes reflexive bowel movement and defecation after a meal.

5.5.1.1. Intravenous Pharmaceutical Compositions

LCRF1-35 infused intravenously was as effective and potent as when given intraduodenally (FIG. 8B). This indicates that i.v. LCRF stimulates CCK release, because LCRF does not stimulate the pancreas directly as indicated by its lack of effect on amylase release from isolated pancreatic acini. Because i.v. administered LCRF can stimulate CCK release, the i.v. route of administration may be useful in some situations and be superior to i.v. infusion of CCK itself, for the reasons described above, because LCRF stimulates the release of endogenous, natural cholecystokinin.

The situations in which i.v. rather than oral administration might be warranted are in patients in which the oral route is impractical or difficult, such as in patients (adults and children) receiving intravenous feedings because of bowel surgery or bowel dysfunction. They frequently develop gallstones because of lack of stimulation of the gallbladder, and this can be prevented by intravenous administration of CCK-8.

For intravenous administration, LCRF could be supplied in sterile vials for injection or for drip infusion. Based on animal studies, the dose rate for human intravenous infusion would be expected to be in the range of 0.1-1.0 μg/kg body weight/hr. This is less than for oral route because there is no digestive enzyme inactivation of the peptide infused intravenously.

5.5.2 Control of Insulin Secretion

LCRF compositions are contemplated to be useful for the stimulation of insulin secretion. CCK has been demonstrated to potentiate amino acid-induced insulin secretion in humans. Therefore, in conditions in which insulin secretion is deficient, such as type I or II diabetes mellitus, CCK may be useful, and therefore a

CCK-releasing peptide that is orally active, such as LCRF, will be valuable. In this case, LCRF may be administered orally in compositions as described above.

In early stages of type II diabetes, insulin secretion is in excess due to insulin insensitivity. It is considered desirable to reduce hyperinsulinemia in type II diabetes, and it has been shown that endogenous and exogenous CCK in humans can reduce hyperinsulenima by slowing the emptying of carbohydrate from the stomach.

5.5.3 Regulation of Gastric Emptying

Gastric emptying in humans is regulated by CCK, and that both CCK and trypsin inhibitors slow gastric emptying in diabetic patients who have abnormally rapid gastric emptying. This is important because rapid gastric emptying is now recognized as a symptom of early diabetes, and it exacerbates postprandial hyperglycemia and hyperinsulinemia.

Diabetic subjects, both type I (insulin-dependent) and type II (adult onset, non-insulin dependent), would benefit from LCRF by taking it prior to and with high carbohydrate meals, as this type of meal empties the fastest in such subjects. For example, a diabetic subject may take LCRF as a pre-load in a liquid vehicle 10-20 minutes prior to a meal to slow the gastric emptying of the subsequent meal. This would also be expected to reduce food intake, as gastric distention is an important factor in satiety. If a high carbohydrate, high calorie beverage is being consumed, it would be recommended that LCRF, as a powder, be mixed in with the beverage to slow its emptying from the stomach and enhance its satiety value.

5.5.4 Reduction in Gallbladder Stasis (increased gallbladder emptying)

Gallbladder stasis is a completion of diminished food, especially fat, in the intestine, as in people on weight reduction diets, and absence of food in the intestine, as in patients on total parenteral nutrition. This leads to gallstones in many cases. In

the former case, subjects on low fat, low calorie weight reduction regimens would be advised to take LCRF prior to each meal, to enhance the ability of that meal to release CCK and thereby more fully contract the gallbladder. More frequent contraction of the gallbladder by exogenous CCK is known to prevent gallstones in susceptible subjects, and it would therefore be expected that LCRF taken orally would do likewise.

5.5.5 Appetite Suppression and Control of Food Intake.

To test the ability of LCRF to induce satiety and reduce food consumption, a recognized experimental design for testing the effect of endogenous CCK on food intake was employed. In this procedure, young rats approximately 12 days old were removed from their nest and weighed. They were then rapidly infused intragastrically with 1 ml of isotonic saline (control) or $LCRF_{1-35}$ in saline. They were then re weighed and housed in a groups at 33° C. Ten minutes later they were transferred to individual containers at room temperature and allowed access to 4 ml of milk diet (commercial half and half) for 30 min. After the test, rats were dried and weighed, and the mil intake was expressed as the percent of body weight gained during the test (%BWG) Two separate studies were carried out with separate sets of rats, but using the same preparation of $LCRF_{1-35}$.

TABLE 5

Dose	N	Mean	SD	Min	Max
0.0 (saline)	11	1.5136	0.81426	0.39	3.17
	10	1.438	0.88547	0.10	2.80
3.0 μg LCRF	11	1.18818	0.76448	0.46	2.39
0.0 (saline)	7	1.181857	0.53909	1.2	2.79
1.5 μg LCRF	8	1.63375	0.73455	0.54	2.50
3.0 μg LCRF	8	1.39500	0.58756	0.84	2.32

Linear regression analysis using the SAS statistical analysis system was used to evaluate the dose effect of $LCRF_{1-35}$ on food intake. The data showed: (1) lack of fit: the lack of fit from the liner trend was not significant ($p = >0.30$); (2) the rate of decrease for each μg of dose was 0.11% BWG, and 0.14 % BWG. The linear trend for decreasing food intake is found to be highly significant, in both experiments, with $p<0.001$. These experiments establish in a mammalian model that LCRF acts as a satiety agent at very low doses to reduce food intake.

Use of LCRF for reduction of food intake in humans. LCRF is expected to reduce food intake in the above experiment because previous studies in humans showed that soybean trypsin inhibitor suppressed food intake. It has been proposed that LCRF mediates the stimulation of CCK release by trypsin inhibitor. Because oral trypsin inhibitors also increase CCK release in humans and reduce food intake in humans, it is expected that LCRF will stimulate CCK release and reduce food intake in humans.

LCRF, incorporated into the compositions described previously for oral delivery, would be taken prior to a meal to induce and augment the "pre-load" phenomenon that helps reduce food intake normally. It would be expected that the

LCRF preparation would be taken prior to each large meal, and prior to or with highly calorie-rich liquid beverages, *e.g.*, cola beverages. Maximum induction of the satiety actions of LCRF would be achieved by taking LCRF 10-20 minutes prior to a meal, and once again just prior to or with the meal. The dosage of LCRF would depend on the form taken, e.g., enteric coated or as a powder. LCRF would not be taken in-between meals, as it acts to augment the satiety value of foods, but may not have less satiety actions if given alone.

5.6 Example 6

LCRF Variants and fragments have been previously described. Several of the variants and truncated species have been assessed and found to have biological activity. Examples include, but are not limited to $LCRF_{1-6}$, $LCRF_{1-35}$, $LCRF_{7-23}$, $LCRF_{1-37}$ and $LCRF_{1-35}$, Lys→ala at position 19).

5.6.1 $LCRF_{1-35}$ Bioactivity

The N-terminus sequence of LCRF including amino acids 1-35 was synthesized. The peptide significantly stimulated pancreatic protein and fluid secretion in conscious rats when infused either intravenously or intraduodenally. Intraduodenal infusion significantly stimulated increased plasma CCK concentration but had no effect on amylase release from pancreatic acini. The CCKA-receptor antagonist MK329 abolished the pancreatic stimulatory activity. Under similar conditions, DBI 1-86 and DBI 33-50 did not significantly stimulate pancreatic secretion. Trypsin-digestion abolished the CCK-releasing activity of $LCRF_{1-35}$.

5.6.1.2 Pancreatic secretory response to intraduodenal infusion of Monitor Peptide and native purified LCRF

The dose/response relationships between incremental protein and fluid output in rats infused with recombinant monitor peptide and native LCRF are illustrated in FIG. 6A and 6B. Monitor peptide and native LCRF significantly stimulated pancreatic protein and fluid secretion at doses of 1-2 μg, respectively, with fluid output closely paralleling protein output. Both peptides exhibited supramaximal inhibition at higher doses in this mode.

5.6.1.3 Pancreatic secretory response to intraduodenal infusion of $LCRF_{1-35}$

The dose/relationships between incremental pancreatic protein and fluid output with $LCRF_{1-35}$ and $LCRF_{1-6}$ (as control) are illustrated in FIG. 7A and 7B. $LCRF_{1-35}$ significantly stimulated protein secretion at doses from 0.1 to 0.5 μg/rat, with peak response at 0.1 μg. Fluid output followed a similar dose response curve. $LCRF_{1-6}$ did not stimulate pancreatic protein or fluid secretion.

5.6.1.4 Comparison between intravenous vs. intraduodenao routes for stimulation of pancreatic secretion by $LCRF_{1-35}$

FIG. 8A and 8B illustrates the comparison between i.v. vs. i.d. routes of administration of $LCRF_{1-35}$. The dose-response curve was quite similar via both routes, with peak response occurring at the same dose, 0.1 μg, via either route. These results indicate $LCRF_{1-35}$ infused intravenously may have access to CCK secreting cells of the small intestine, since other results, described below, show that $LCRF_{1-35}$ does not stimulate pancreatic secretion directly.

5.6.1.5 Pancreatic secretory response to various subfragments of $LCRF_{1-35}$

To determine the minimal fragment of LCRF possessing CCK-releasing activity, several fragments within the sequence of $LCRF_{1-35}$ were synthesized and tested, using the "bioassay" model. As illustrated in FIG. 9, only fragment $LCRF_{11-25}$ significantly stimulated pancreatic protein secretion, with increased potency but decreased efficacy compared to $LCRF_{1-35}$.

5.6.1.6 Pancreatic secretory response to intraduodenal infusion of diazepam binding inhibitor (DBI) and DBI fragment and GRP

These studies were carried out in the "bioassay model", described in Example 2. Across a wide dose range (FIG. 10A and 10B), none of the peptides significantly stimulated pancreatic protein or fluid secretion, under conditions in which $LCRF_{1-35}$ and native LCRF strongly stimulated pancreatic secretion. This result indicates that the peptide, diazepam binding inhibitor, reported to be a CCK-releasing peptide in the rat by Herzig, *et al.* (1995), does not stimulate CCK release in conscious rats fully recovered from surgery. These results indicate that DBI does not mediate feedback regulation of CCK release in the rat, contrary to claims by Herzig, *et al.*

5.6.1.7 Effect of CCK receptor blockade on the pancreatic secretory response to intraduodenal $LCRF_{1-35}$ and effect of intraduodenal $LCRF_{1-35}$ on plasma CCK concentration

These studies were carried out in a physiological model, *i.e.*, with bile and pancreatic juice returned to the intestine. FIG. 11A and 11B show the time course of pancreatic protein and fluid secretion during continuous intraduodenal infusion of 25 μg of $LCRF_{1-35}$ and saline control for 2 hours, and the effect of the CCK receptor antagonist MK329 on the response to $LCRF_{1-35}$. $LCRF_{1-35}$ significantly stimulated pancreatic fluid and protein secretion, compared to basal, and this response was abolished by MK329. The incremental pancreatic protein and fluid responses are

illustrated in FIG. 12A and 12B. FIG. 13 illustrates the plasma CCK responses in the same experiments, determined on blood samples withdrawn 60 minutes after the start of infusion of the test compounds. $LCRF_{1-35}$ significantly increased plasma CCK concentration compared to basal levels with NaCl or $LCRF_{1-6}$. Basal levels of plasma CCK were higher than previously reported in rates with 100% of pancreatic juice returned to the intestine, possibly because partial return of pancreatic juice does not completely suppress spontaneous secretion of CCK under these conditions. The results illustrated in FIGS. NO. 11-13 strongly indicate that the stimulation of pancreatic secretion by $LCRF_{1-35}$ is mediated by release of CCK.

5.6.1.8 Effect of tryptic digestion of $LCRF_{1-35}$ on CCK-releasing activity

FIG. 14 illustrates the effect of incubation of $LCRF_{1-35}$ with purified bovine trypsin (1mg/ml) at 37° C for 24 hours. Control LCRF indicates $LCRF_{1-35}$ incubated under the same conditions but without trypsin. Trypsin Control consisted of a solution of trypsin incubated under the same conditions but without $LCRF_{1-35}$. Tryptic digestion completely abolished the pancreatic secretory response to $LCRF_{1-35}$. Trypsin Control did not contain any residual trypsin activity, insuring that the lack of effect of $LCRF_{1-35}$ incubated with trypsin was not due to a suppressive effect of trypsin on pancreatic secretion. This result shows that $LCRF_{1-35}$ meets the requirement for a trypsin-sensitive CCK-releasing peptide secreted by the intestine and has activity similar to that of the native polypeptide.

5.6.1.9 Effect of $LCRF_{1-35}$ on CCK secretion by dispersed rate intestinal mucosal cells *in vitro*

FIG. 15 illustrates the dose-response relationship of CCK release to $LCRF_{1-35}$ in dispersed rat intestinal cells. $LCRF_{1-35}$ significantly increased CCK release, compared to basal release, at 5 nM and 50 nM concentrations of $LCRF_{1-35}$. These results show that $LCRF_{1-35}$ directly stimulates CCK release from intestinal mucosal

cells, presumably from CCK "I" cells, and may mediate the indirect stimulation caused by nutrients in the same system.

5.6.2 $LCRF_{1-35}$ Immunoneutralization

Immunoneutralization of LCRF inhibits the pancreatic secretory and CCK response to diversion of bile-pancreatic juice and peptone infusion

Peptone stimulates pancreatic secretion when infused intraduodenally in absence of pancreatic juice in the intestine, and this response is mediated by CCK and by endogenous LCRF. To determine whether endogenous LCRF truly mediates this response, the effect of duodenal peptone infusion on pancreatic secretion was tested in rats infused concomitantly intraduodenally with purified IgG (antiserum #22322) obtained from rats immunized with $LCRF_{7-23}$ (Quality Controlled Biochemicals, Inc., Hopkinton, MA). As illustrated in FIG. 16A and 16B, anti-LCRF IgG infused simultaneously with 5% peptone completely abolished the pancreatic secretory response to this nutrient solution. Control rabbit IgG from unimmunized rabbit plasma had no inhibitory effect on the pancreatic secretory response to peptone under the same conditions. These results strongly indicate that the pancreatic secretory response to peptone is mediated by LCRF.

To determine the role of LCRF in the pancreatic secretory and plasma CCK responses to diversion of bile-pancreatic juice in the rat, a different antisera was used. Antisera were raised in rabbits to the fragment $LCRF_{22-37}$. This antisera was used without further purification. Antisera, 0.1 ml, were injected i.v. in rats ~ 1 hour prior to diversion of bile-pancreatic juice from the duodenum. The results were compared to results obtained in the same rats the day before who had received 0.1 ml. NRS in similar manner. The results are illustrated in FIGS. 17A, 17B and 18.

Diversion of bile-pancreatic juice significantly stimulated pancreatic protein and fluid secretion in both groups. To determine whether the LCRF antiserum inhibited this response, as would be predicted, the increment (output above basal) was calculated and the peak responses for each group compared. These results (inserts in FIG. 17A and 17B) show that the LCRF antisera (LCRF Ab) significantly inhibited the pancreatic fluid and protein responses to diversion of bile-pancreatic juice.

FIG. 18 illustrates the plasma CCK responses in the same experiment, determined on blood samples withdrawn 30 minutes after diversion of bile-pancreatic juice. LCRF antiserum significantly suppressed plasma CCK concentrations, compared to rats receiving no antiserum and compared to rats receiving NRS. The results of this experiment strongly indicate that LCRF mediates, in part, the pancreatic secretory and plasma CCK responses to bile-pancreatic juice diversion.

FIG. 19 illustrates the lack of direct effect of $LCRF_{1-35}$ on pancreatic cells. Isolated pancreatic acini were incubated with increasing concentrations of CCK-8 or $LCRF_{1-35}$ and amylase release into the medium measured. $LCRF_{1-35}$ had no effect onamylase release at concentrations at which CCK-8 dose-dependently increased amylase release. These results indicated that $LCRF_{2-35}$ does not directly stimulate the pancreas. Therefore the stimulation of pancreatic secretion by i.d. and i.v. $LCRF_{1-35}$ is probably indirect, via release of CCK.

5.6.3 LCRF Fragments and Epitopes

The smallest LCRF fragment with full LCRF agonist activity will be determined. This biological activity will be determined with the *in vivo* and/or *in vitro* test described above. Because LCRF activity is destroyed by the proteolytic activity of trypsin and because there are only three trypsin sensitive sites (two lysines and one arginine) initial fragment screening will be conducted around these basic

amino acid residues. Peptides having approximately 30 amino acids with a centered lysine or arginine will be prepared, based upon the LCRF sequence already known or to be determined. When the active fragment is identified, the link to peptide surrounding the basic amino acids will be shortened systematically. After each shortening, biological activity will be determined until full biological activity with a minimal size fragment is determined. Once this is done, then the central basic amino acid may be replaced by an amino acid such as, *e.g.*, homoarginine that results in a peptide not sensitive to hydrolysis by trypsin but retaining biological activity. Alternatively, arginine or lysine may be substituted by a nonbasic amino acid. The final step will be to assure that the trypsin insensitive fragment also has the biological CCK-releasing activity desired.

It is understood, of course, that non-peptide LCRF analogs of the minimally sized active fragment may be prepared by methods well known to those of skill in the art. Such non-peptide bonds may eliminate the need to replace the basic amino acid signaling trypsin sensitivity.

* * * * *

The following references are incorporated in pertinent part by reference herein for the reasons cited above.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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(B) STREET: 201 West 7th Street

(C) CITY: Austin

(D) STATE: Texas

(E) COUNTRY: USA

(F) POSTAL CODE (ZIP): 78701

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(B) STREET: 011 Allen Building

(C) CITY: Durham

(D) STATE: North Carolina

(E) COUNTRY: USA

(F) POSTAL CODE (ZIP): 27708

(ii) TITLE OF INVENTION: LUMINAL CHOLECYSTOKININ-RELEASING FACTOR

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/005,872

(B) FILING DATE: 26-OCT-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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1 5 10 15

Tyr Gln Lys Tyr Glu His Thr Ser Ser Pro Ser Gln Leu Leu Ala Pro
20 25 30

Gly Asp Tyr Pro Cys Val Ile Glu Val
35 40

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 123 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(A) NAME/KEY: modified_base
(B) LOCATION:3..123
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "N = T, A, C or G"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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GAACATACNT GNTGNCCNTG NCAATTNTTN GCNCCNGGNG ATTATCCNTG TGTNATTGAA 120

GTN 123

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ser Thr Phe Trp Ala Tyr Gln Pro Asp Gly Asp Asn Asp Pro Thr Asp
1 5 10 15

Tyr Gln Lys Tyr Glu His Thr Ser Ser Pro Ser Gln Leu Leu Ala Pro
20 25 30

Gly Asp Tyr
35

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:3..21
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "Y = T or C"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:9..18
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "N = Inosine"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:15..16
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "R = A or G"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTYTGGGCNT AYCARCCNGA YGG 23

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:3..18

(D) OTHER INFORMATION:/mod_base= OTHER
/note= "Y = T or C"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:9..15
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "H = A, C or T"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:12..13
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "R = A or G"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTYTGGGCHC ARCCHGAYGG 20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:3..21
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "Y = T or C"

(ix) FEATURE:
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(B) LOCATION:12..15
(D) OTHER INFORMATION:/mod_base= OTHER
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(A) NAME/KEY: modified_base
(B) LOCATION:3..9
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "R = A or G"

(ix) FEATURE:
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(B) LOCATION:6..15
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "Y = C or T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTRTGYTCRT AYTTYTG 17

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) LOCATION:3..21
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "N = Inosine"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:9..18
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "R = A or G"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCNATNACRC ANGGRTARTC NCC 23

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(A) NAME/KEY: modified_base
(B) LOCATION:3..12
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "D = G, A or T"

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(A) NAME/KEY: modified_base
(B) LOCATION:6..7
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "S = G or C"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:9..21
(D) OTHER INFORMATION:/mod_base= OTHER
/note= "R = A or G"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:24..25
(D) OTHER INFORMATION:/mod_base= OTHER
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCDATSACRC ADGGRGGRTA RTCNCC 26

CLAIMS:

1. An isolated cholecystokinin-releasing polypeptide which specifically binds with antibodies raised against a polypeptide having at least the amino acid sequence of SEQ ID NO: 1.

2. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO:1.

3. The polypeptide of claim 2 further defined as having a mass as determined by mass spectrometry of about 8136 daltons.

4. The polypeptide of claim 1 that is isolated from luminal secretions of small intestine.

5. The polypeptide of claim 2 that stimulates cholecystokinin release.

6. The polypeptide of claim 1 that has the amino acid sequence of SEQ ID NO: 1.

7. The polypeptide of claim 1 further defined as having at least 85% homology to the amino acid sequence of SEQ ID NO: 1.

8. An isolated cholecystokinin releasing polypeptide comprising:

a) the amino acid sequence of SEQ ID NO: 1; or

b) the amino acid sequence of SEQ ID NO: 1 from position 1 to position 35; or

c) the amino acid sequence of SEQ ID NO: 1 from position 11 to position 25; or

d) the amino acid sequence of SEQ ID NO:1 from position 1 to position 6; or

e) the amino acid sequence of SEQ ID NO:1 from position 7 to position 23; or

f) the amino acid sequence of SEQ ID NO:1 from position 22 to position 37; or

g) the amino acid sequence of SEQ ID NO:1 from position 1-35 where Lysine is replaced with alanine at position 19; or

h) functional or homologous variants thereof.

9. A composition comprising the polypeptide of claim 1 or claim 2.

10. The composition of claim 9 further defined as comprising a physiologically acceptable excipient.

11. A purified antibody that specifically binds to the polypeptide of claim 2.

12. The antibody of claim 11 wherein the antibody is linked to a detectable label.

13. A method of generating an immune response, comprising administering to a mammal a pharmaceutical composition comprising an immunologically effective amount of the composition of claim 9.

14. A method for detecting luminal cholecystokinin-releasing peptide of claim 8 in a biological sample, comprising the steps of:

a) obtaining a biological sample suspected of containing a luminal cholecystokinin releasing peptide;

b) contacting said sample with a first antibody that binds to the protein or peptide of claim 8 under conditions effective to allow formation of an immune complex; and

c) detecting the immune complex so formed.

15. An immunodetection kit comprising, in suitable container means, one or more protein or polypeptides as defined by claim 8, or an antibody that binds to a protein or peptide as defined by claim 8, and an immunodetection reagent.

16. An isolated nucleic acid segment that encodes a cholecystokinin-releasing polypeptide which specifically binds with antibodies raised against a polypeptide having at least the partial amino acid sequence of SEQ ID NO: 1.

17. An isolated nucleic acid segment that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:1.

18. The nucleic acid segment of claim 16 or claim 17 further defined as comprising the nucleic acid sequence of SEQ ID NO: 2 or the complement thereof or a sequence which hybridizes to SEQ ID NO: 2 under conditions of high stringency.

19. The nucleic acid segment of claim 16 or claim 17 wherein the encoded polypeptide has the amino acid sequence of SEQ ID NO:1.

20. The nucleic acid segment of claim 16 or claim 17 further defined as an RNA segment.

21. A recombinant vector comprising a DNA segment which comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:1.

22. A recombinant vector comprising a DNA segment which comprises a cholecystokinin-releasing polypeptide that specifically binds with antibodies raised against a polypeptide having at least the partial amino acid sequence of SEQ ID NO: 1.

23. The recombinant vector of claim 21 or 22 wherein said DNA segment comprises a nucleotide sequence in accordance with SEQ ID NO: 2.

24. A recombinant host cell comprising a recombinant vector in accordance with claim 21 or claim 22.

25. The recombinant host cell of claim 24 wherein the host cell is *S. mutans*.

26. A method of suppressing appetite comprising:

providing a composition in accordance with claim 10; and

administering said composition to a subject in need thereof in an amount effective to suppress appetite.

27. A method for stimulating gallbladder contraction or treating gallbladder disease related to gallstone formation, the method comprising:

providing a composition in accordance with claim 10; and

administering said composition to a subject in need thereof in an amount effective to stimulate gallbladder emptying.

28. A method of inhibiting gastric emptying, the method comprising:

providing a composition in accordance with claim 10; and

administering said composition to a subject in need thereof in an amount effective to.

delay gastric emptying.

29. A method of stimulating insulin secretion comprising:

providing a composition in accordance with claim 10; and

administering said composition to a subject in need thereof in an amount effective to stimulate insulin secretion.

30. A method of preparing an orally administerable preparation useful to suppress appetite, stimulate gallbladder emptying, inhibit stomach emptying, or stimulate insulin secretion, the method comprising formulating an orally acceptable preparation comprising a therapeutically effective amount of the polypeptide of claim .1 or claim 2.

31. A method of using a DNA segment that includes an isolated cholecystokinin-releasing gene encoding the polypeptide of claim 1 or claim 2, comprising the steps of:

a) preparing a recombinant vector in which a cholecystokinin-releasing gene encoding the polypeptide of claim 1 or claim 2 is positioned under the control of a promoter;

b) introducing said recombinant vector into a recombinant host cell;

c) culturing the recombinant host cell under conditions effective to allow expression of an encoded cholecystokinin-releasing protein or peptide; and

d) collecting said said expressed cholecystokinin-releasing protein or peptide.

Fig. 1.

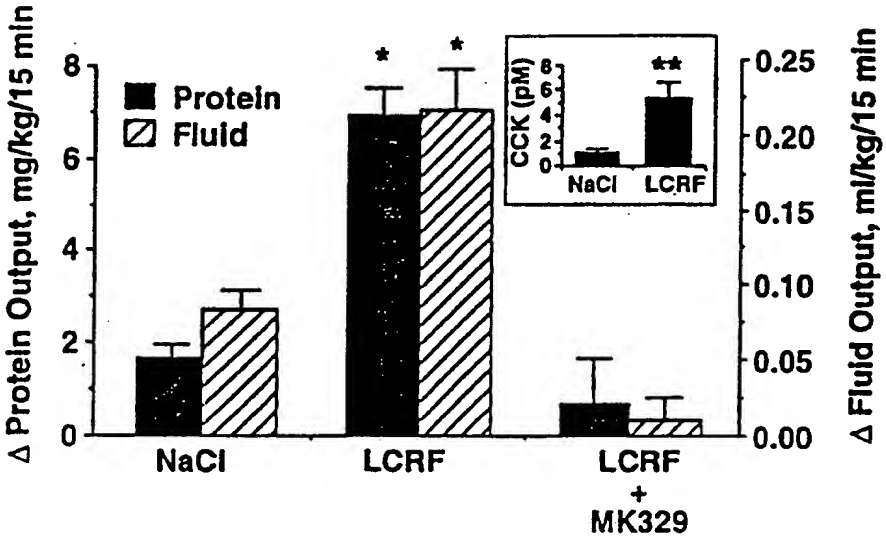

Δ Protein Output, mg/kg/15 min
Δ Fluid Output, ml/kg/15 min
Protein
Fluid
CCK (pM)
NaCl
LCRF
NaCl
LCRF
LCRF + MK329

Fig. 2.

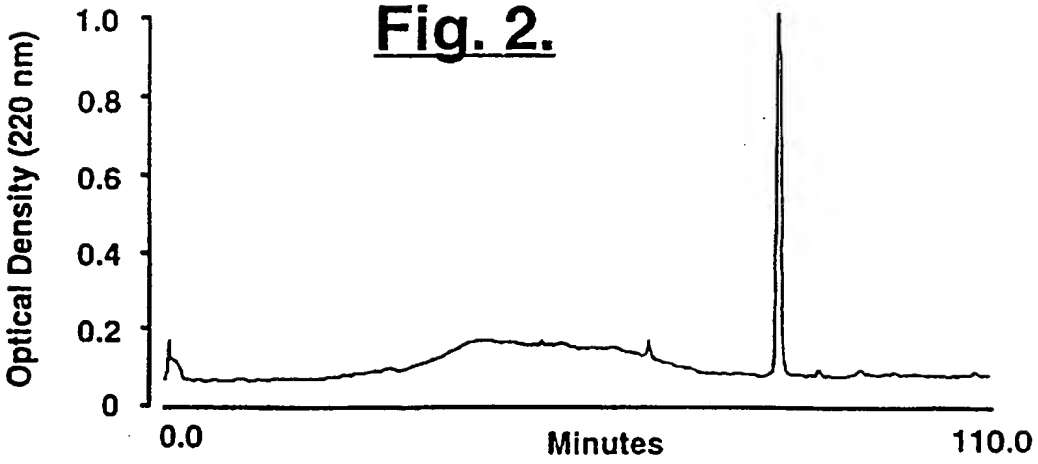

Optical Density (220 nm)
Minutes
0.0
110.0

Fig. 3.

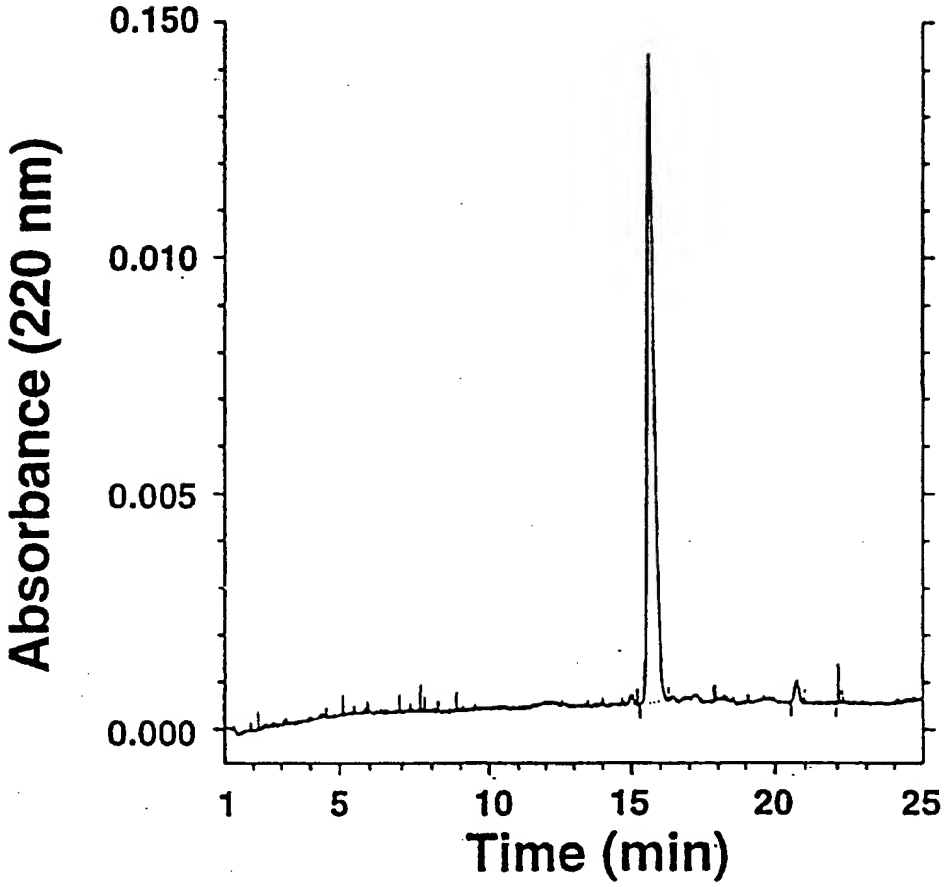


Fig. 4.

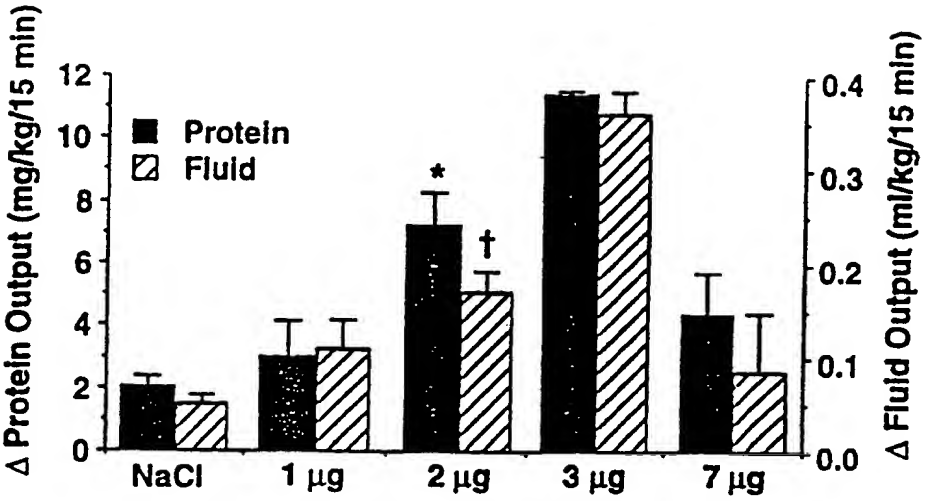
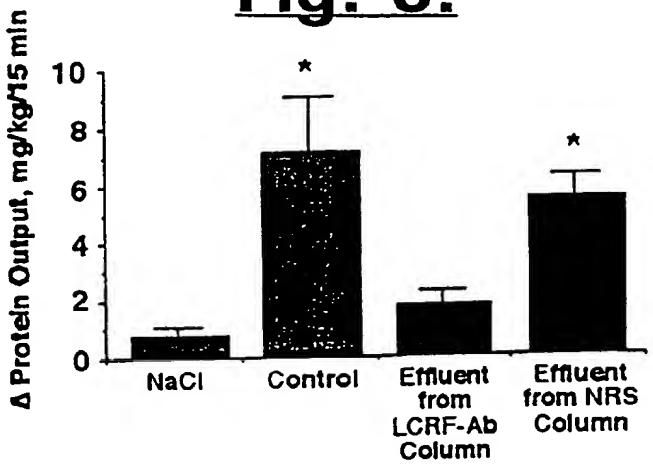

Δ Protein Output (mg/kg/15 min)
Δ Fluid Output (ml/kg/15 min)
Protein
Fluid
0
2
4
6
8
10
12
0.0
0.1
0.2
0.3
0.4
*
†
NaCl
1 µg
2 µg
3 µg
7 µg

Fig. 5.


Δ Protein Output, mg/kg/15 min
0
2
4
6
8
10
*
*
NaCl
Control
Effluent from LCRF-Ab Column
Effluent from NRS Column

Comparison Between Monitor Peptide and Native LCRF on Pancreatic Secretion

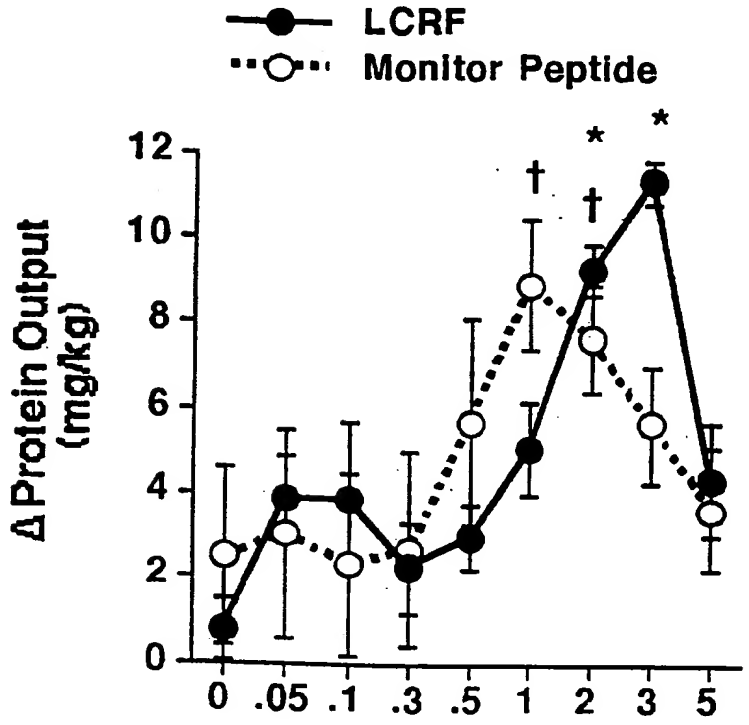


FIG. 6A

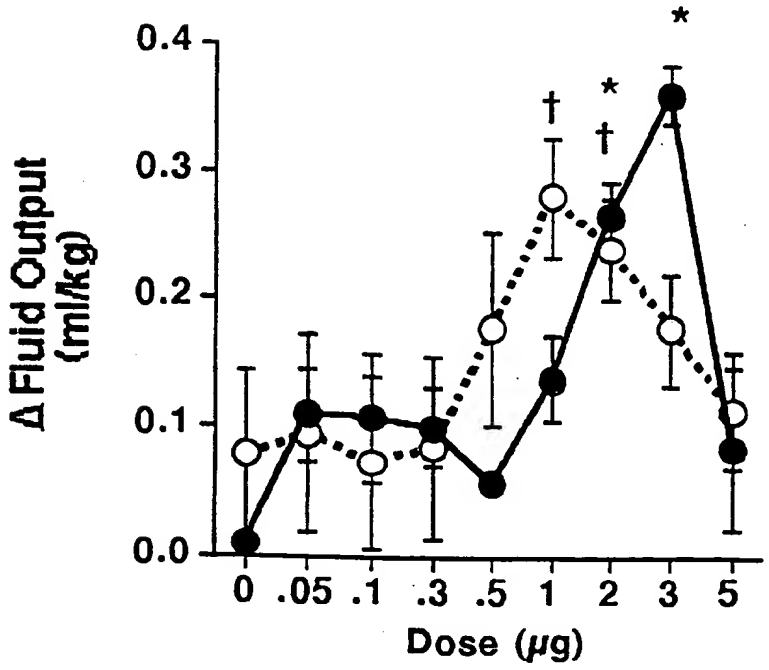


FIG. 6B

Dose-response Relationship between Intraduodenal LCRF[1-35] and Pancreatic Protein and Fluid Secretion

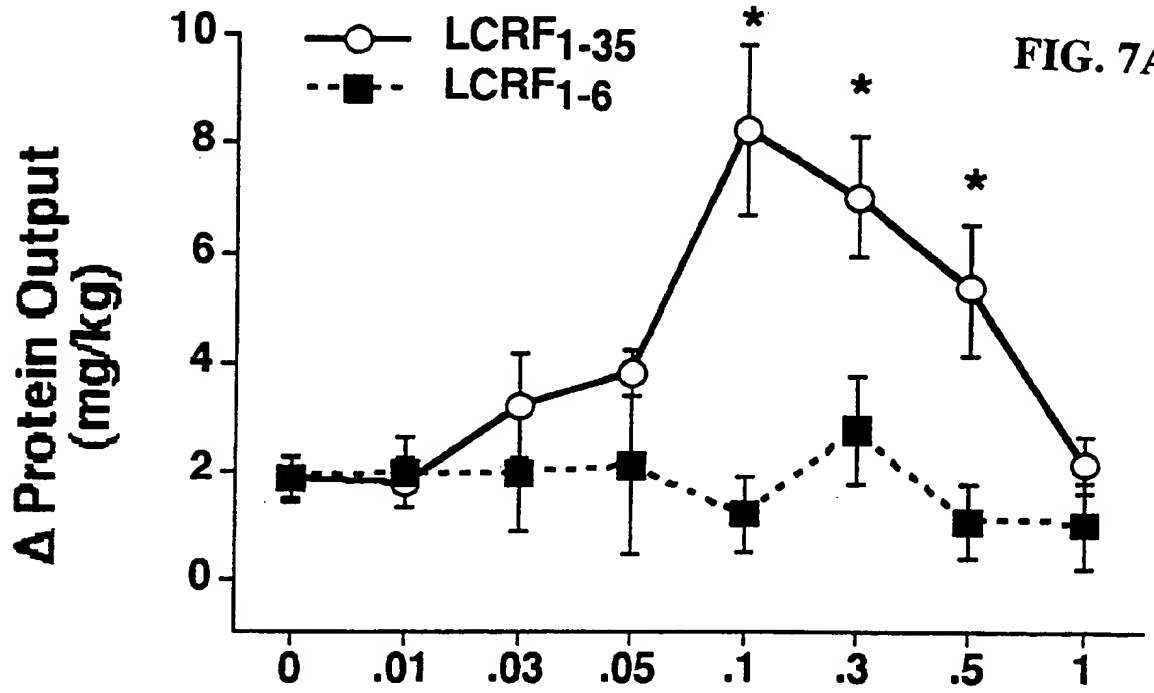


FIG. 7A

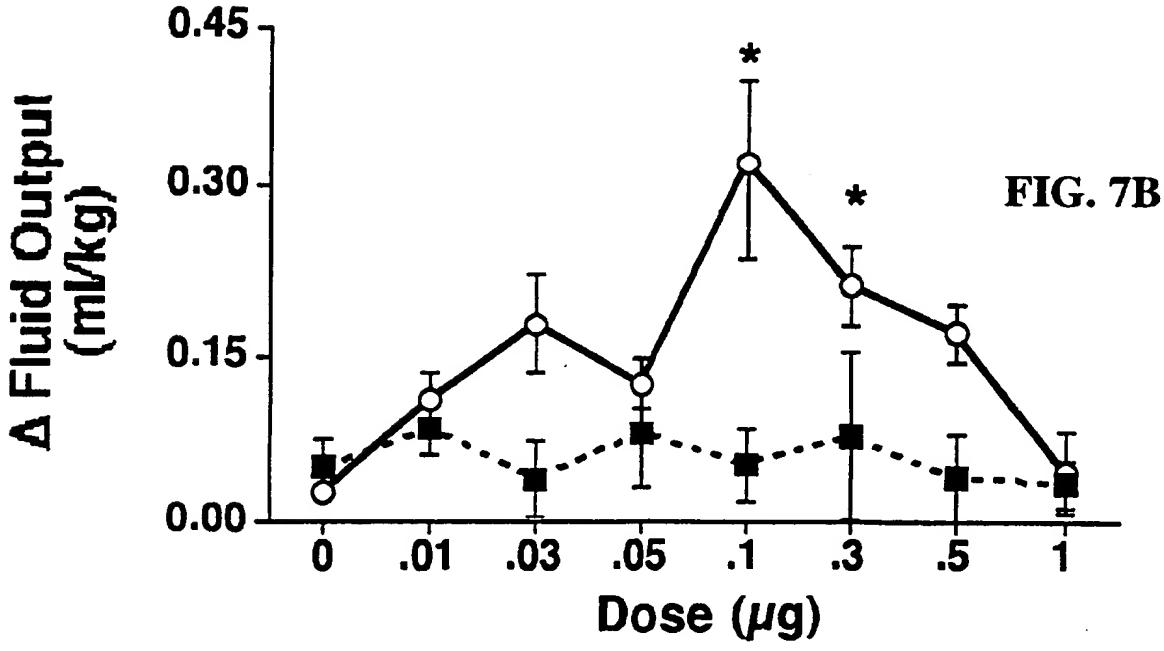


FIG. 7B

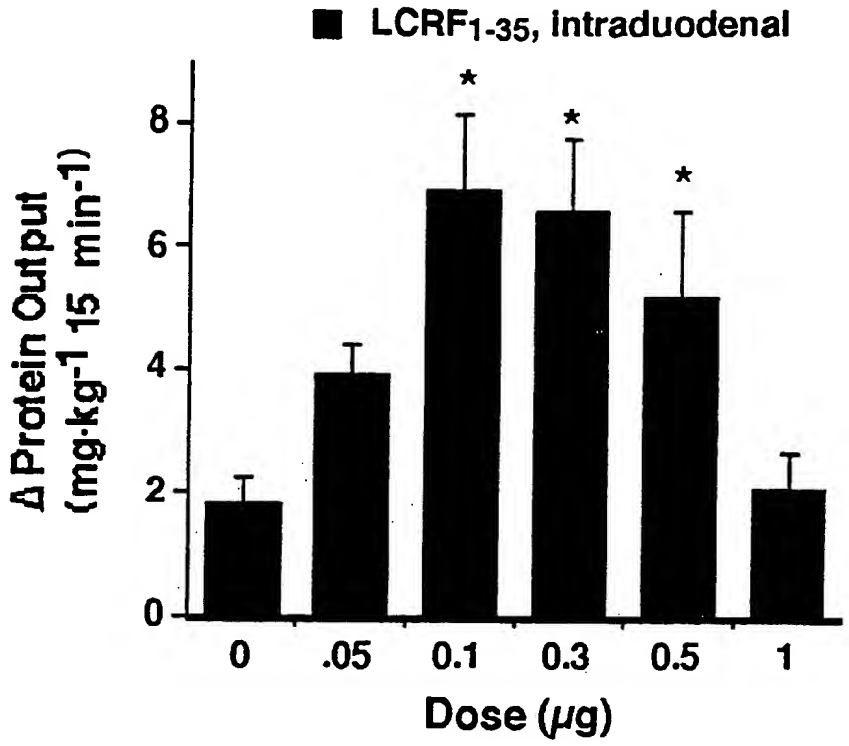

Comparison Between i.d. vs. i.v.
LCRF1-35 on Pancreatic Secretion
LCRF1-35, intraduodenal
Δ Protein Output
(mg·kg-1 15 min-1)
0
2
4
6
8
*
*
*
0
.05
0.1
0.3
0.5
1
Dose (μg)

FIG. 8A

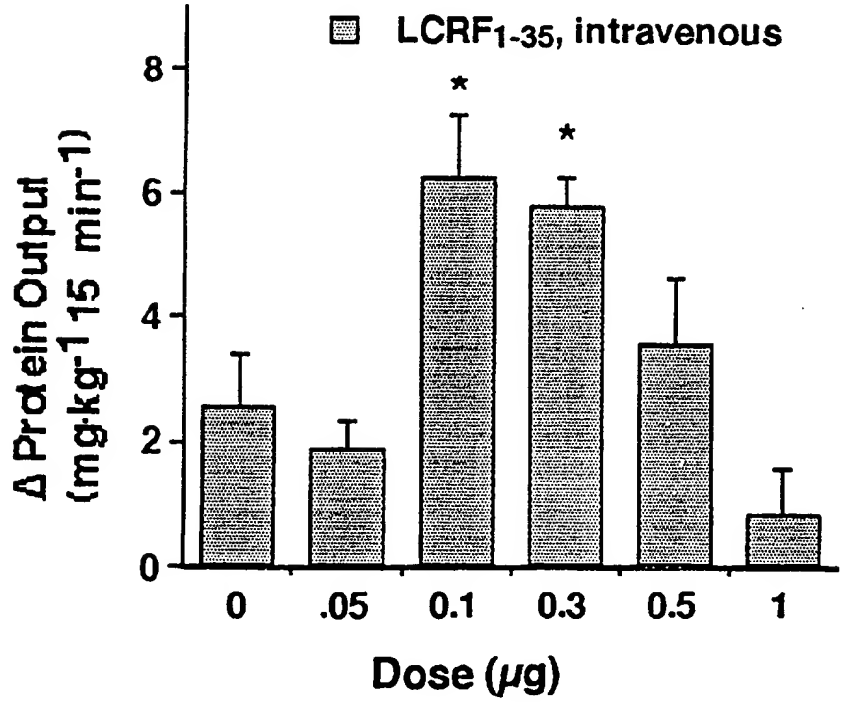

LCRF1-35, intravenous
Δ Protein Output
(mg·kg-1 15 min-1)
0
2
4
6
8
*
*
0
.05
0.1
0.3
0.5
1
Dose (μg)

FIG. 8B

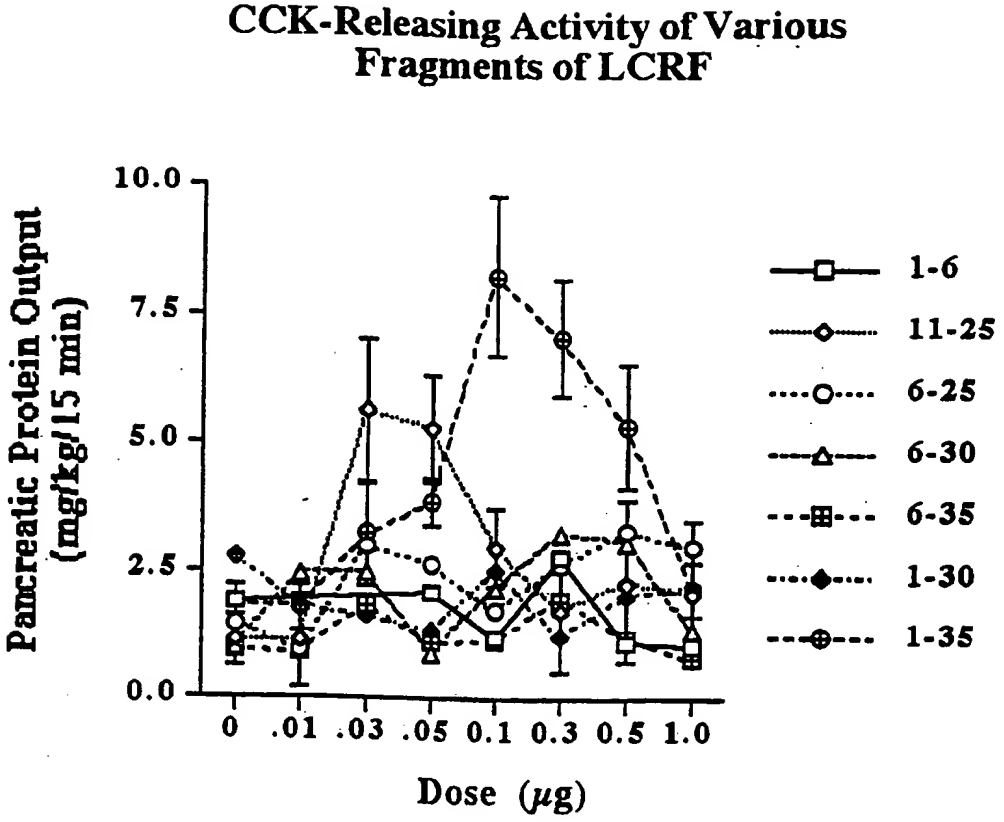

CCK-Releasing Activity of Various Fragments of LCRF
Pancreatic Protein Output (mg/kg/15 min)
10.0
7.5
5.0
2.5
0.0
0
.01
.03
.05
0.1
0.3
0.5
1.0
Dose (μg)
1-6
11-25
6-25
6-30
6-35
1-30
1-35

FIG. 9

Absence of CCK-Releasing Activity of Diazepam Binding Inhibitor

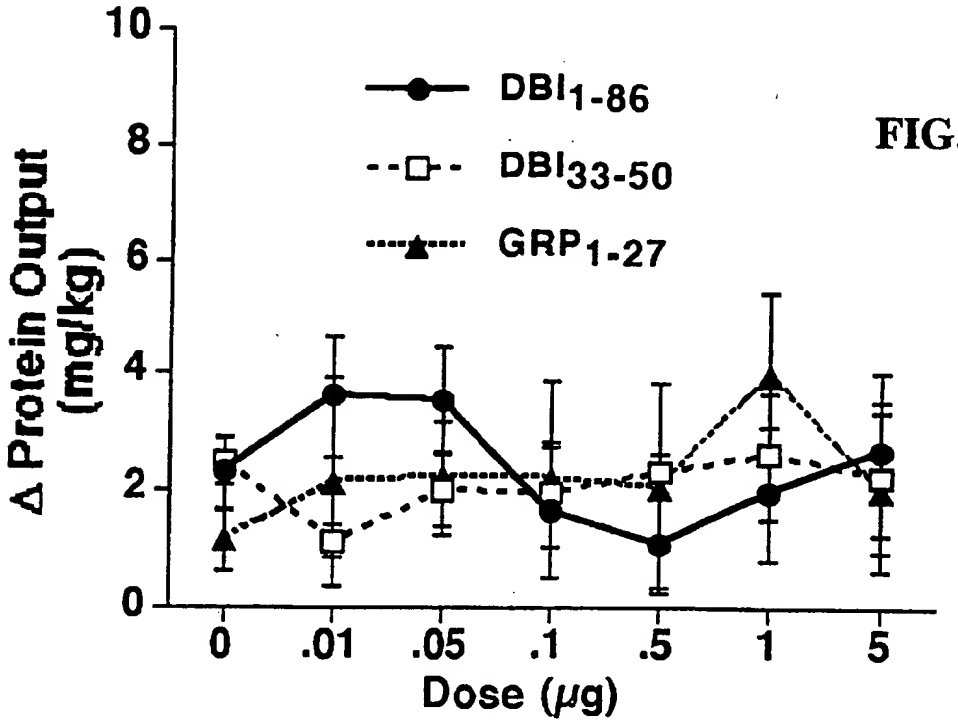


FIG. 10A

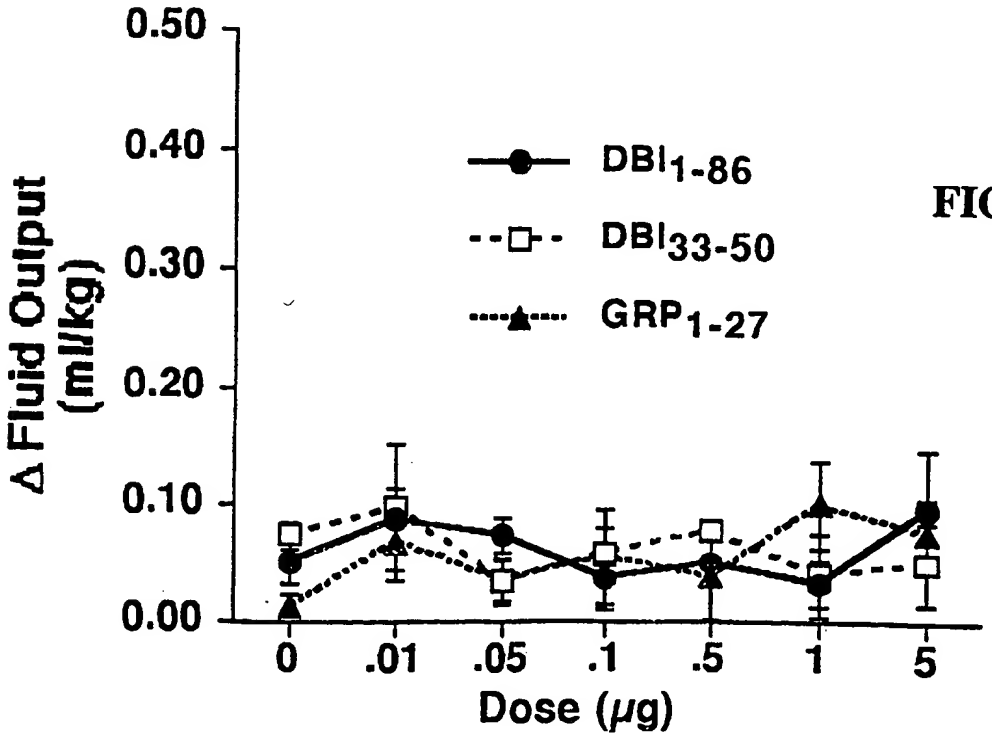


FIG. 10B

Effect of CCK-Receptor Blockade on $LCRF_{1-35}$ Stimulation of Pancreatic Secretion

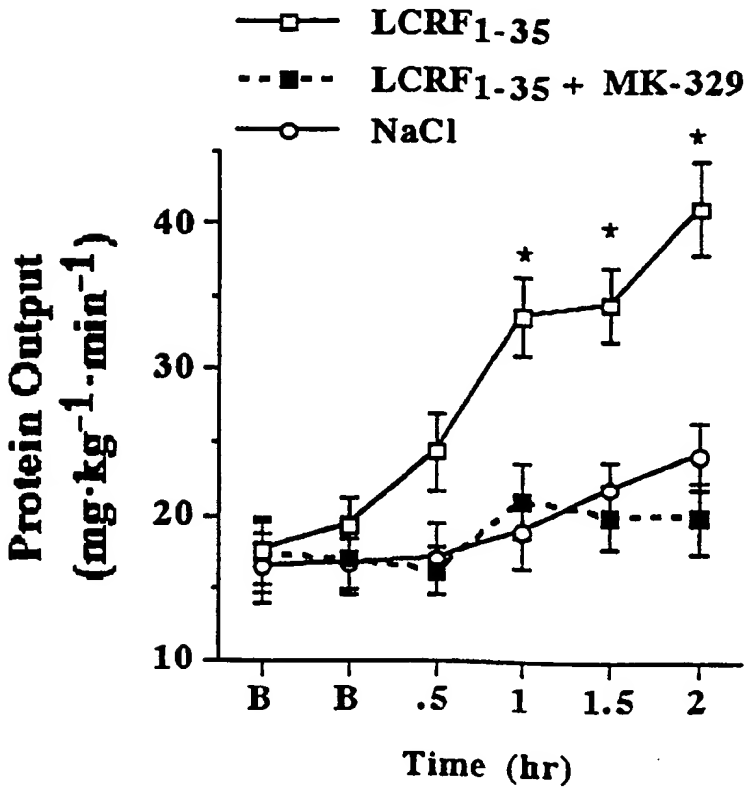


FIG. 11A

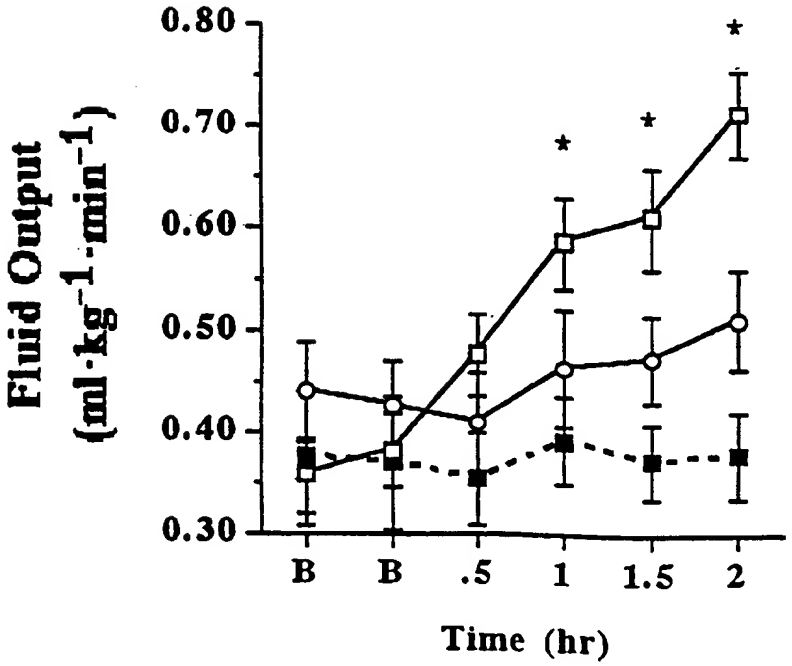


FIG. 11B

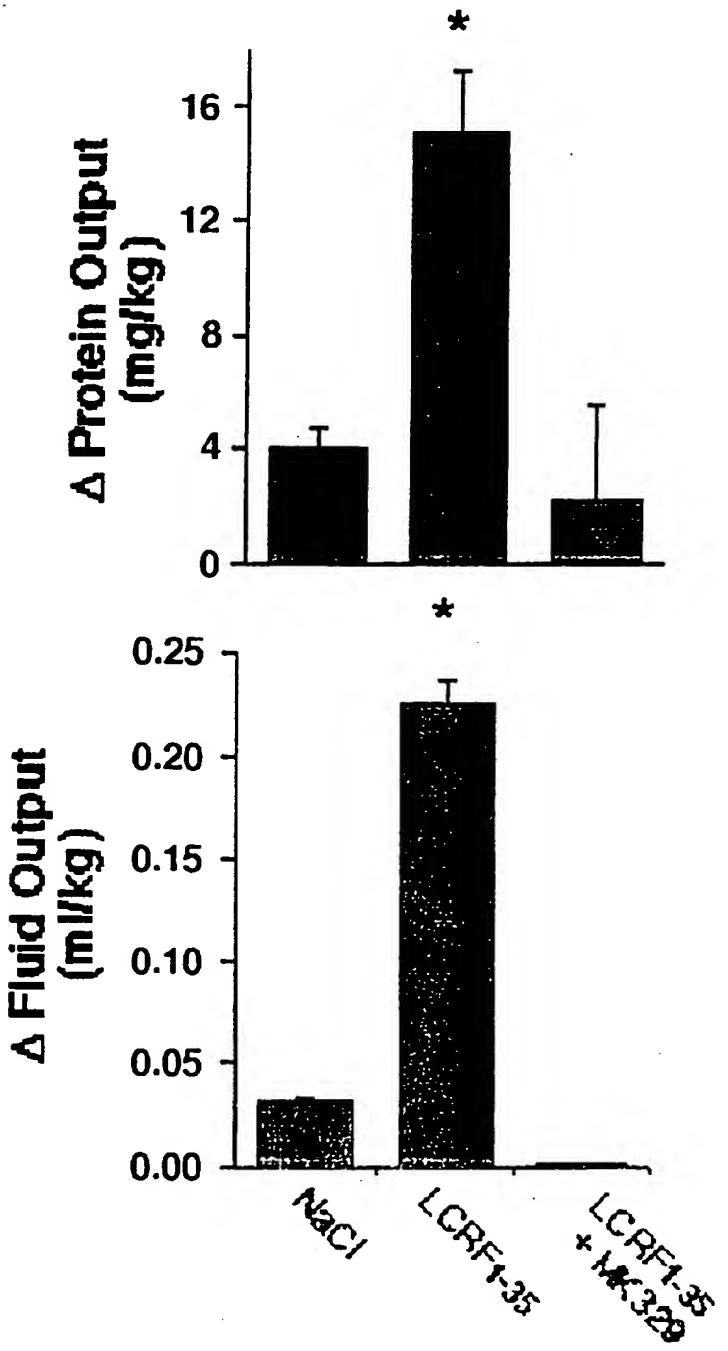


FIG. 12A

FIG. 12B

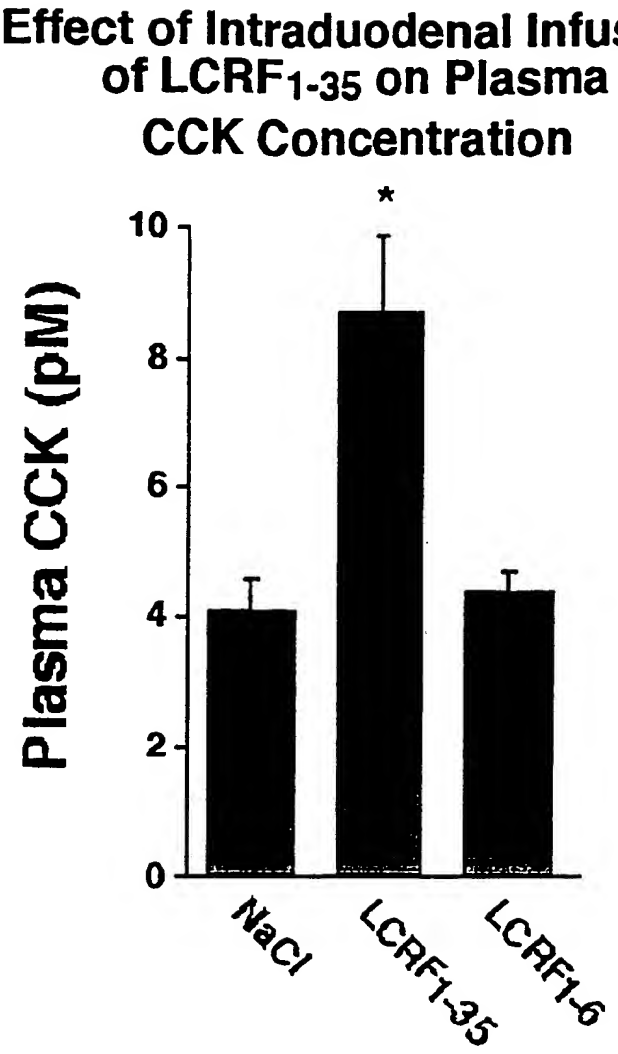

Effect of Intraduodenal Infusion of LCRF1-35 on Plasma CCK Concentration
Plasma CCK (pM)
10
8
6
4
2
0
*
NaCl
LCRF1-35
LCRF1-6

FIG. 13

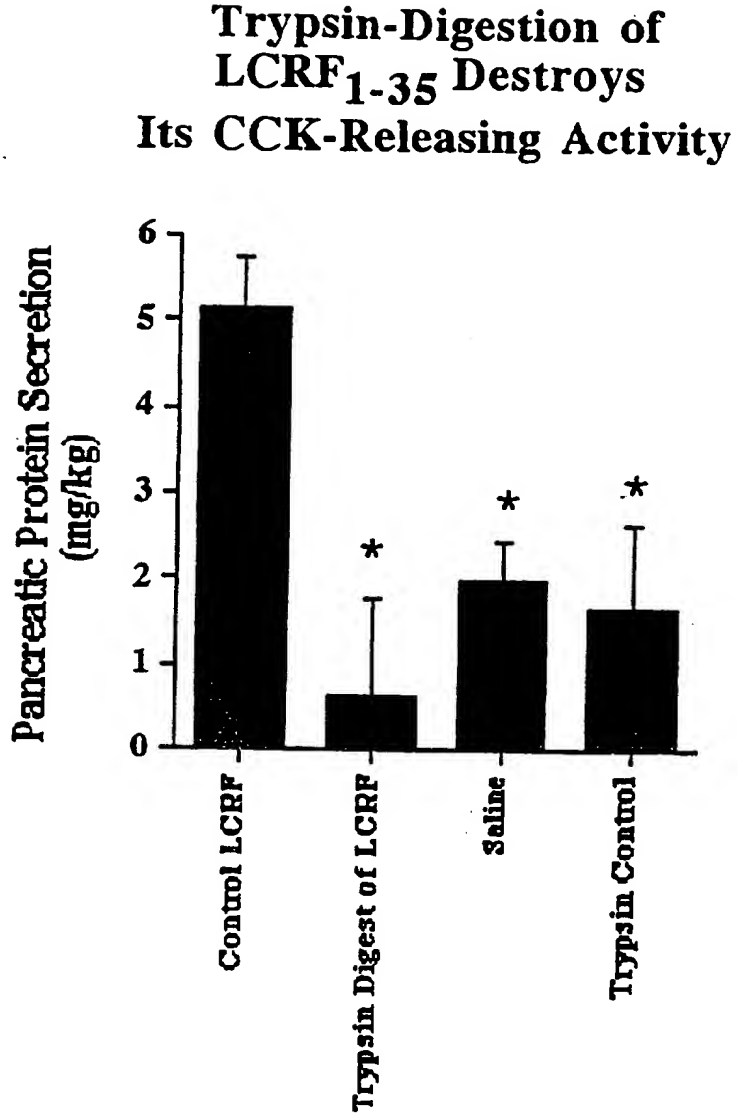

Trypsin-Digestion of
LCRF1-35 Destroys
Its CCK-Releasing Activity
Pancreatic Protein Secretion
(mg/kg)
0
1
2
3
4
5
6
*
*
*
Control LCRF
Trypsin Digest of LCRF
Saline
Trypsin Control

FIG. 14

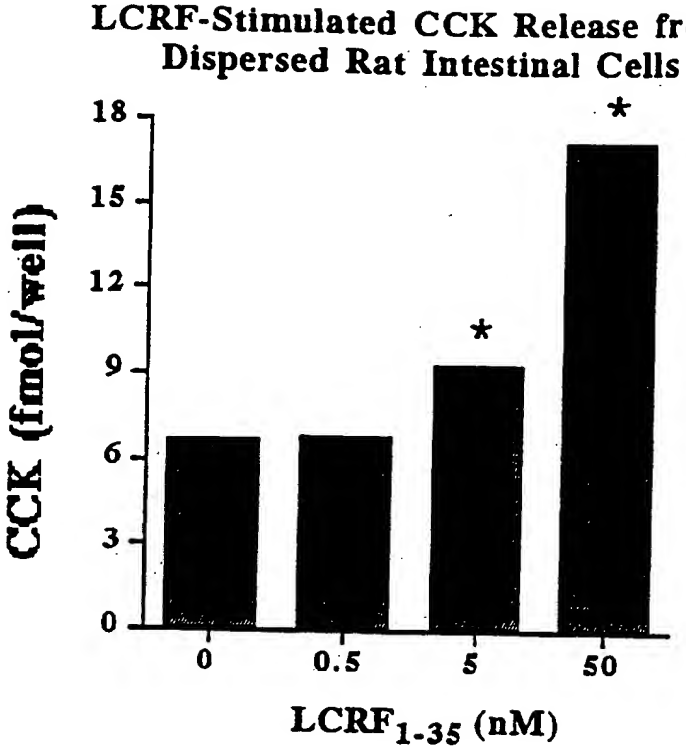

LCRF-Stimulated CCK Release from Dispersed Rat Intestinal Cells
CCK (fmol/well)
18
15
12
9
6
3
0
*
*
0
0.5
5
50
LCRF1-35 (nM)

FIG. 15

Immunoneutralization of LCRF: Anti-LCRF IgG Abolishes the Pancreatic Response to Intraduodenal Peptone

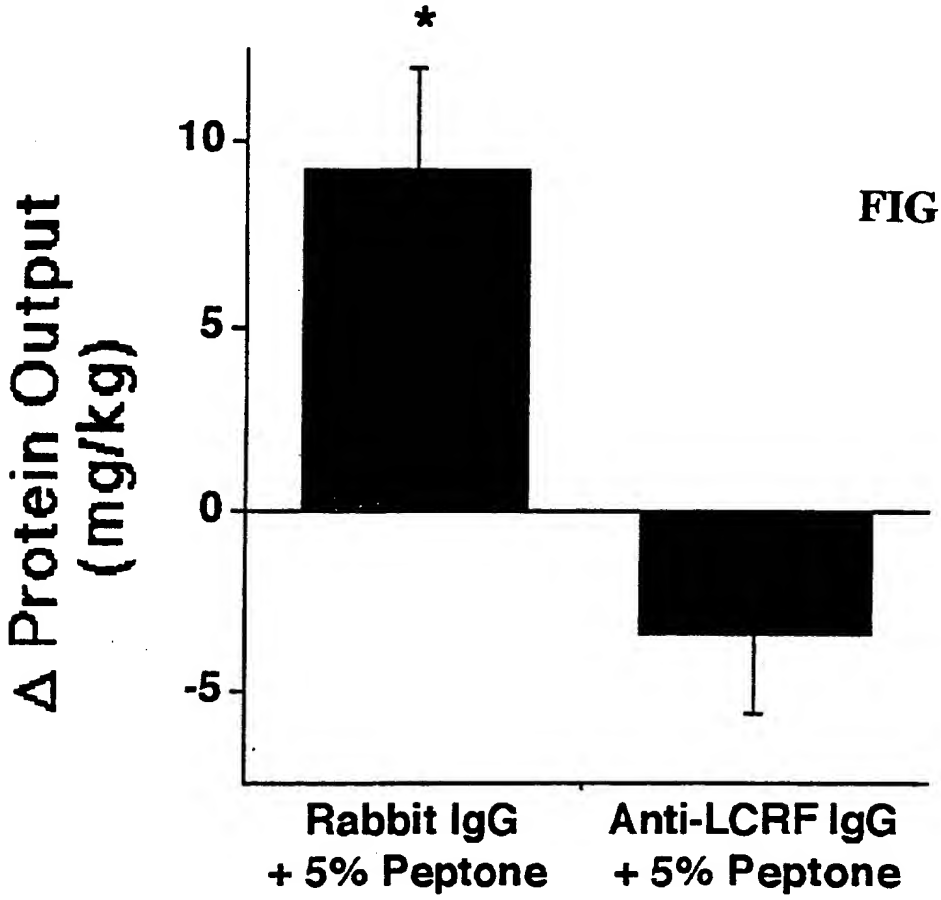


FIG. 16A

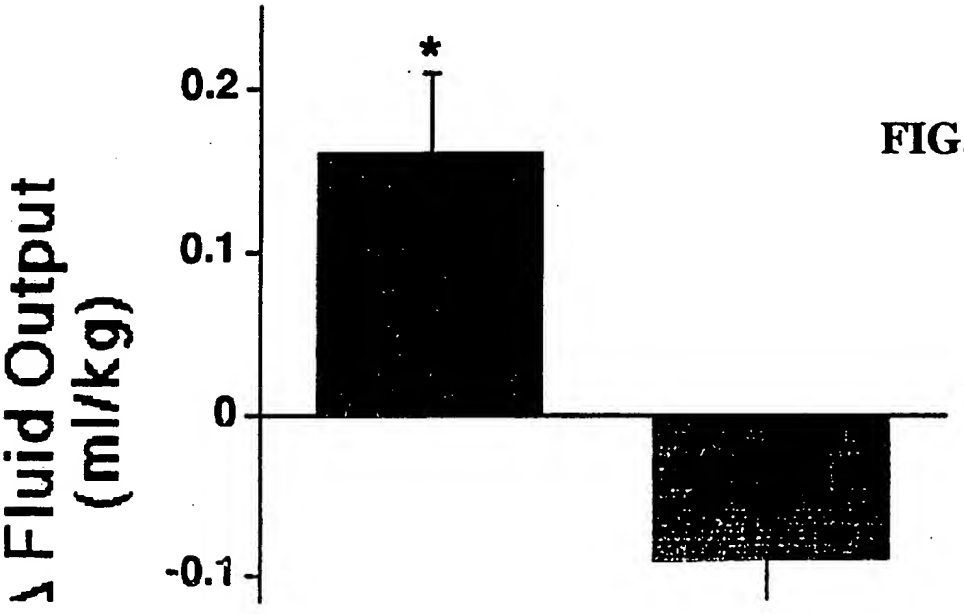


FIG. 16B

Effect of LCRF Antiserum on Pancreatic Response to Bile-Pancreatic Juice Diversion

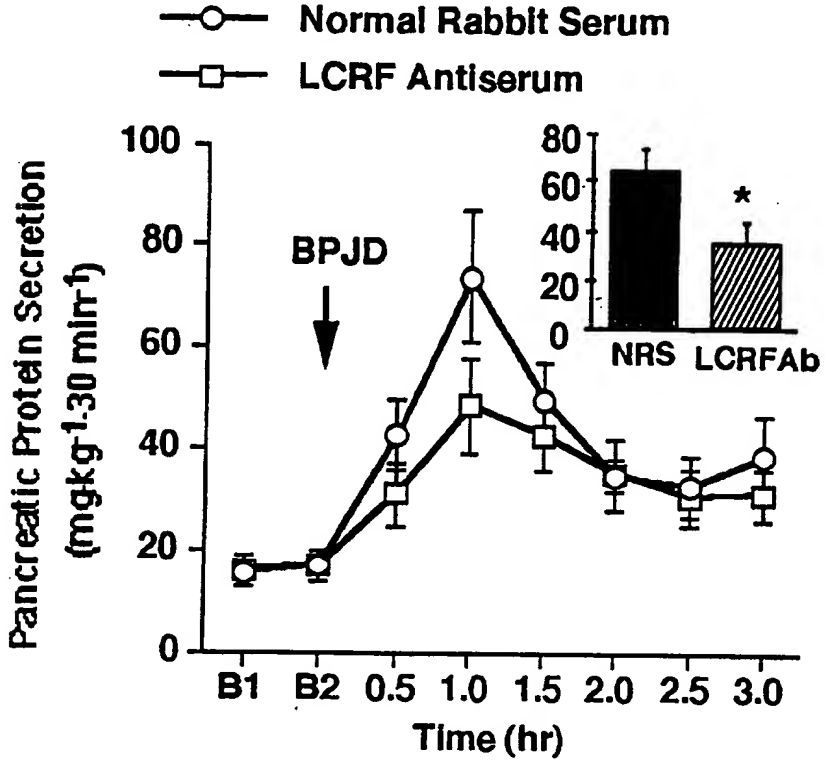


FIG. 17A

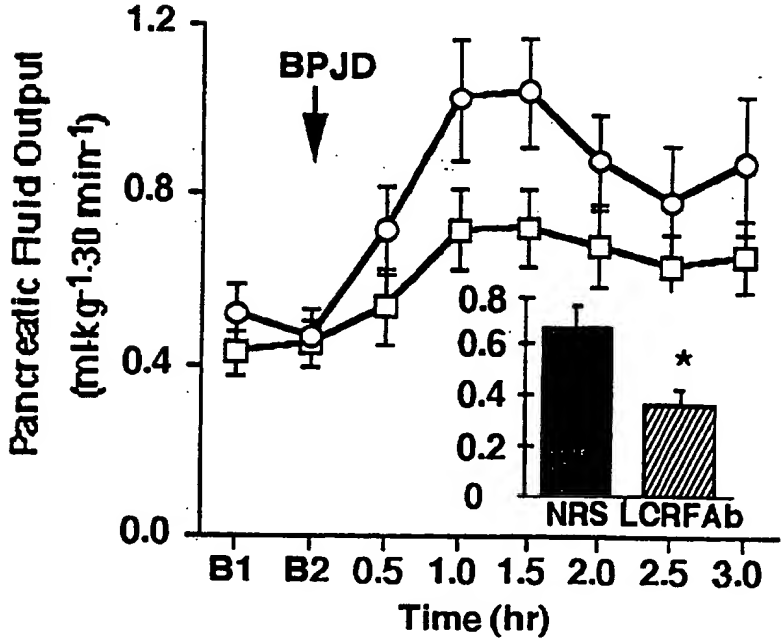


FIG. 17B

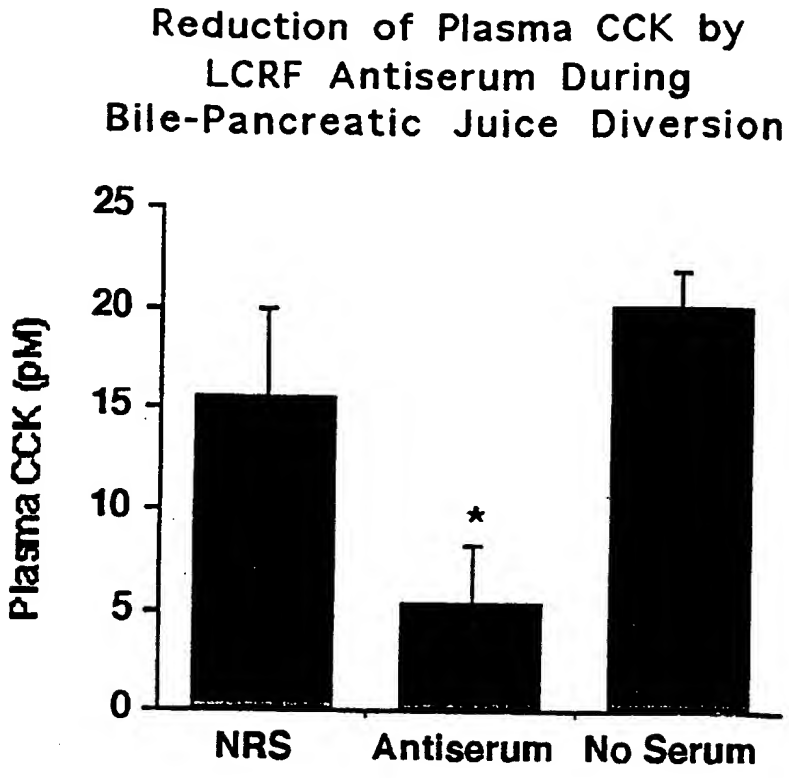

Reduction of Plasma CCK by
LCRF Antiserum During
Bile-Pancreatic Juice Diversion
Plasma CCK (pM)
25
20
15
10
5
0
*
NRS
Antiserum
No Serum

FIG. 18

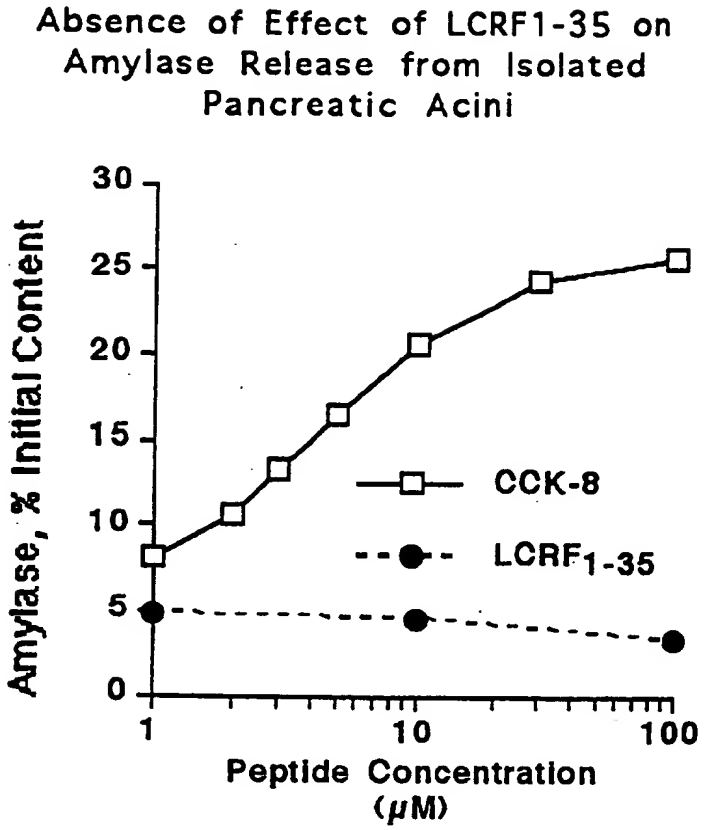

Absence of Effect of LCRF1-35 on Amylase Release from Isolated Pancreatic Acini
Amylase, % Initial Content
30
25
20
15
10
5
0
1
10
100
Peptide Concentration (μM)
CCK-8
LCRF1-35

FIG. 19

FIG. 20A

FIG. 20B

FIG. 21A

FIG. 21B

FIG. 22A

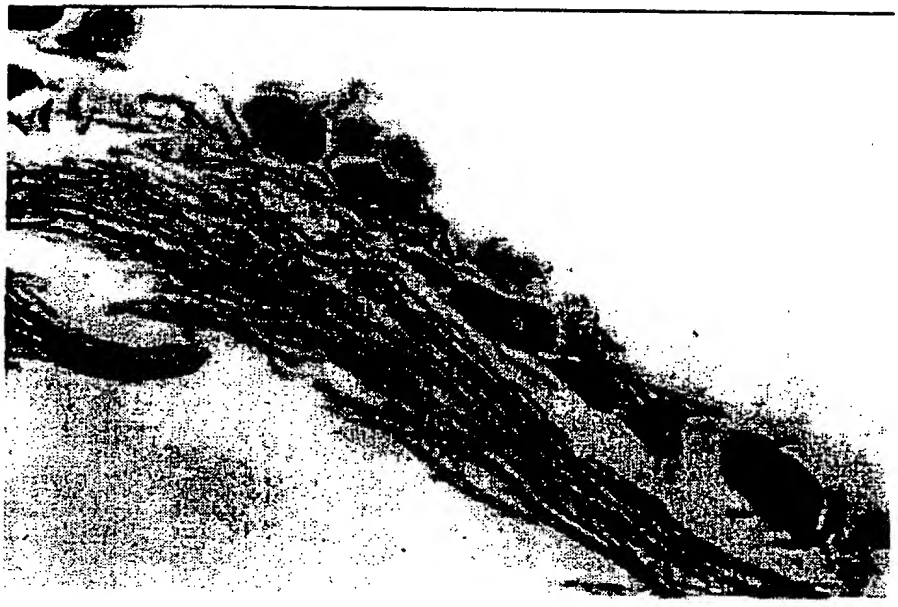


FIG. 22B

FIG. 23A

FIG. 23B

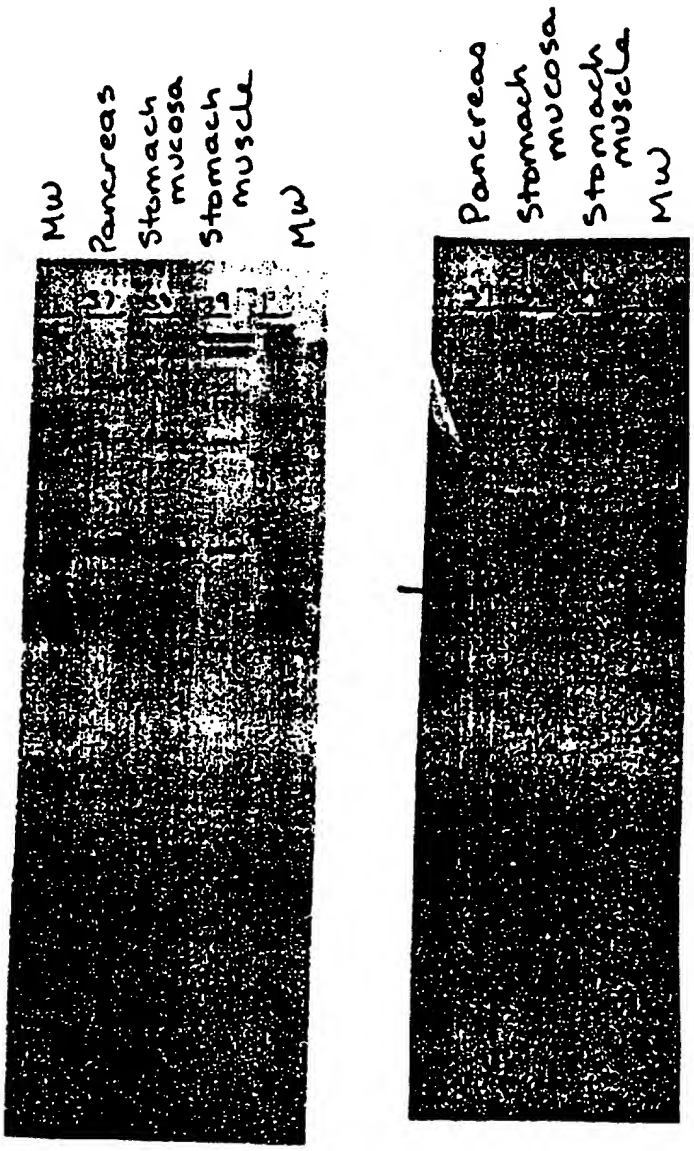

MW
Pancreas
Stomach mucosa
Stomach muscle
MW
Pancreas
Stomach mucosa
Stomach muscle
MW
NRS
QPDG

FIG. 24

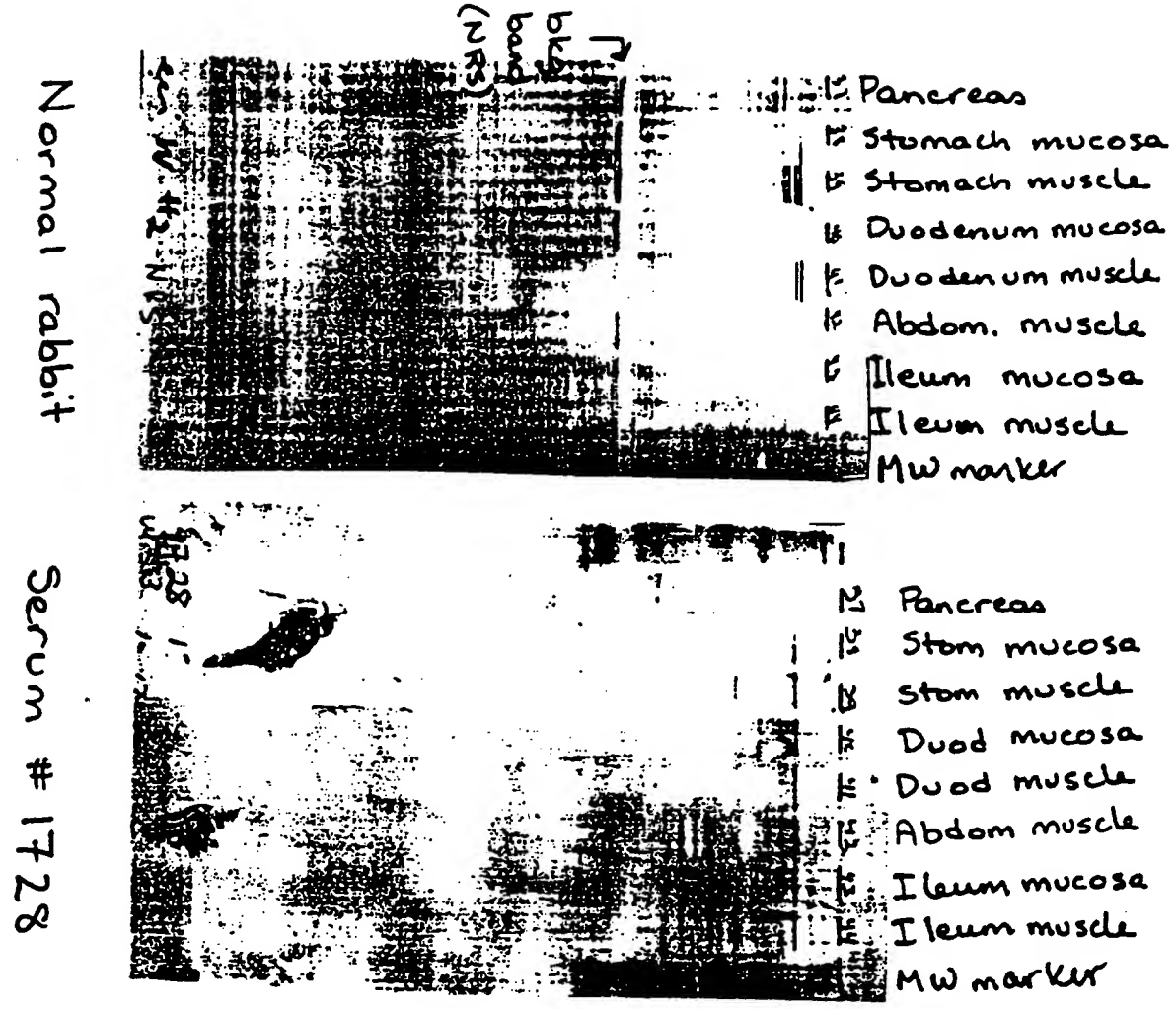

Normal rabbit
Serum # 1728
blue band (NRS)
Pancreas
Stomach mucosa
Stomach muscle
Duodenum mucosa
Duodenum muscle
Abdom. muscle
Ileum mucosa
Ileum muscle
MW marker
Pancreas
Stom mucosa
Stom muscle
Duod mucosa
Duod muscle
Abdom muscle
Ileum mucosa
Ileum muscle
MW marker

FIG. 25

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 96/17998

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/16 C12N15/19 C12N15/74 C12N5/10 C12N1/21
C07K14/52 C07K14/575 C07K16/24 C07K16/26 A61K38/19
A61K38/22 G01N33/53 //C12R1:46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 262, no. 19, 5 July 1987, pages 8956-8959, XP002028440 IWAI K. ET AL.: "Purification and sequencing of a trypsin-sensitive cholecystokinin-releasing peptide from rat pancreatic juice" cited in the application	1,8
A	see the whole document ---	2-7,9-31
A	AMERICAN JOURNAL OF PHYSIOLOGY, vol. 257, no. 20, 1989, pages G175-G181, XP000646810 MIYASAKA, K. ET AL.: "Feedback regulation by trypsin: evidence for intraluminal CCK-releasing peptide" cited in the application see the whole document ---	1-31

-/--

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 April 1997	11. 04. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AMERICAN JOURNAL OF PHYSIOLOGY, vol. 269, no. 3, September 1995, pages G319-G327, XP000647817 LIDDLE R.: "Regulation of cholecystokinin secretion by intraluminal factors" cited in the application see the whole document ---	1-31
A	GUT, vol. 37, no. 2, 1995, page A70 XP000647825 HERZIG K.H. ET AL.: "Diazepam binding inhibitor is a CCK releasing peptide in the intestine" cited in the application see the whole document ---	1-31
P,X	PNAS, U.S.A, vol. 93, no. 9, 30 April 1996, pages 4415-4420, XP002028441 SPANNAGEL A. ET AL.: "Purification and characterization of a luminal cholecystokinin-releasing factor from rat intestinal secretion" see the whole document -----	1-8, 11-25,31

3

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 96/ 17998

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13 (part.),26-29
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13 (partially) and 26-29 (as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.